British Journal of Pharmacology (2001) 134, 1367–1374

www.nature.com/bjp

Effects of sepsis on mast cells in rat dura mater: influence of L-NAME and VIP

[1]F. Tore, [2]A.M. Reynier-Rebuffel, [3]N. Tuncel, [4]J. Callebert & *,[1]P. Aubineau

[1]Vascular Pharmacology and Physiopathology, CNRS, UMR 5017, University Bordeaux II, 146, rue Leo Saignat, 33076 Bordeaux, France; [2]Cerebrovascular Research, UPR 646 CNRS, University Paris VII, Paris, France; [3]Department of Physiology, Faculty of Medicine, Osmangazi University, Meselik, 26480, Eskisehir, Turkey and [4]CR Claude Bernard Experimental Pathology and Cellular Communication, Department of Molecular Biology and Biochemistry, Hospital Lariboisiere, Paris, France

1 The influence of lipopolysaccharide (LPS)-induced sepsis on the various mast cell phenotypes of rat dura mater were examined both by immunohistochemical and biochemical methods.

2 Three different populations of mast cells were identified in control rats: connective tissue type mast cells (CTMC) which contain rat mast cell protease1 (RMCP1), histamine, serotonin and heparin, mucosal type mast cells (MMC) which contain RMCP2, histamine and serotonin, and intermediate type which contains both RMCP1 and RMCP2 and probably various proportions of amines and heparin.

3 LPS (25 mg kg^{-1} i.p.) caused changes in the proportions of the various types of mast cells. The number of MMC and intermediate type mast cells significantly increased and the number of mast cells immunopositive for both heparin and serotonin significantly decreased. Biochemical analysis showed that the histamine concentration of dura increased while its serotonin concentration decreased.

4 While vasoactive intestinal peptide (VIP) (25 ng kg^{-1} i.p.) appears to potentiate LPS effects on dura mater mast cells, non-selective inhibition of nitric oxide (NO) synthase by N^g-nitro-L-arginine methyl ester (L-NAME) (30 mg kg^{-1} i.p.) did not influence sepsis-induced mast cell changes.

5 These findings suggest that mast cells of dura mater may play a role in brain protection during sepsis.

British Journal of Pharmacology (2001) **134,** 1367–1374

Keywords: Lipopolysaccharide; sepsis; dura mast cells; rat mast cell proteases; vasoactive intestinal peptide (VIP); L-NAME; histamine; heparin; serotonin

Abbreviations: CNS, central nervous system; CTMC, connective tissue mast cell; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; $IL_{1\beta}$ interleukin 1-beta; IFN-γ, interferon-gamma; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; IR, immuno reactive; LPS, lipopolysaccharide; L-NAME, N^g-nitro-L-arginine methyl ester; MMC, mucosal mast cell; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffer saline; RMCP, rat mast cell protease; TNF-α, tumour necrozing factor-alpha; TRITC, tetramethylrhodamine isothiocynate; VIP, vasoactive intestinal peptide

Introduction

Endotoxemia and sepsis often result in circulatory derangements which manifest as perfusion maldistributions. Inappropriate inflammatory responses which include excessive production of nitric oxide (NO), reactive oxygen species and pro-inflammatory cytokines, over-activation of kallikrein-kinin, complement and coagulation systems, and relaxation of smooth muscles in blood vessel wall and increased endothelial permeability ultimately lead to (or result from) insufficient oxygen supply to tissue, resulting in multiple organ failure (Parillo, 1993; Quan *et al*., 1999; Zervos *et al*., 1999).

The bacterial endotoxin lipopolysaccharide (LPS) is a component of the outer membrane of gram negative bacteria that triggers the synthesis and release of a cascade of immunoregulatory, cytotoxic and pro-inflammatory molecules. Those pro-inflammatory molecules such as tumor necrosis factor-alpha (TNF-α), interleukin 1-beta ($IL_{1\beta}$), interferon-gamma (IFN-γ) cause an increase in expression of inducible nitric oxide synthase (iNOS) in endothelial cells, inflammatory cells. LPS also stimulates iNOS directly. The large amount of NO produced by iNOS has been hypothesized to contribute to LPS-induced hypotension and mortality. However, recent studies showed that NO may have some protective role in sepsis (Kubes & McCafferty, 2000). It has been shown that systemic administration of LPS induces mast cell degranulation and changes their histochemical staining characteristics in the rat (Brown *et al*., 1998; Tuncel *et al*., 2000).

Mast cells are ubiquitous in the body and abundant in close proximity to the microvasculature and nerves. They secrete many vasoactive, nociceptive and proinflammatory molecules

*Author for correspondence at: Laboratoire de physiopathologie et de pharmacologie vasculaire, CNRS, UMR 5017, Universite de Bordeaux II, 146, rue Leo Saignat, 33076 Bordeaux, France.
E-mail: pierre.aubineau@umr5017.u-bordeaux2.fr

in response to various challenges. Each mast cell mediator has more than one function, and the mediators may overlap in their biological effects. They include histamine, serotonin, heparin, pro-inflammatory cytokines, proteases, prostaglandins, NO and neuropeptides such as vasoactive intestinal peptide (VIP) (Galli, 1993; Marshall & Bienenstock, 1994; Reynier-Rebuffel *et al*., 1992; 1994; Theoharides, 1996). Several studies have demonstrated that degranulation of mast cells initiates and modulates a number of important inflammatory cascades (Echtenacher *et al*., 1996; Galli, 1993). A study showed that vascular permeability in an immune complex injury such as reverse passive Arthus reaction is modulated in part by mast cells (Ramos *et al*., 1992). Endogenous agents can activate dura mater mast cells, thereby inducing the release of several classes of mediators that may alter neuronal functions. In addition, dura mast cells as in the many organs can be involved in neuro-immune interactions (Williams *et al*., 1995).

VIP is a multifunctional neuropeptide whose prime immunomodulatory function is anti-inflammatory (Said, 1996). It is synthesized by immune cells including mast cells and can modulate several immune aspects. It has been shown that VIP inhibits cytokine production, proliferation of T cells, macrophage phagocytosis, respiratory burst, and chemotaxis (Delgado *et al*., 1999; Said, 1995). Elevated VIP plasma levels have been reported in humans and animals during septic shock (Brandzaeg *et al*., 1989; Revhaug *et al*., 1988; Said, 1996). This increase may be attributed to a defense mechanism. Moreover, the effects of VIP and non-selective nitric oxide synthase (NOS) inhibition were recently compared in septic rats. VIP increases survival rate and protects tissues by inhibiting mast cell degranulation, and also changes their granular content. Non-selective inhibition of NOS by N^g-nitro-L-arginine methyl ester (L-NAME) decreases survival rate and increases renal damage, possibly due to mast cell degranulation (Tuncel *et al*., 2000; Tuncel & Tore, 1998).

Up to 70% of patients with sepsis and septic shock, develop central nervous system (CNS) symptoms known as septic encephalopathy (Parillo, 1993). The pathophysiology of organ system failure in sepsis, in particular related to the mechanism of septic encephalopathy, is still not fully understood.

Dura mater is considered to be a barrier tissue of the CNS. It is a richly vascularized connective tissue invested by various immune cells including numerous mast cells. It is therefore likely that dura mater fulfills functions other than a simple physical protection of CNS, the presence of many immune cells suggesting a role commonly attributed to lymphoid organs. For example, dura mater mast cells have a prominent role in both septic (meningitis) and sterile inflammatory response (neurogenic inflammation is thought to give rise to migraine headache) (Artico *et al*., 1998; Bergerot *et al*., 2000; Dimitriadou *et al*., 1990; 1997; Moskowitz & MacFarlane, 1993). As in other organs, it is thus possible that the dense population of dura mater mast cells could play an important role during the development of septic encephalopathy. The aim of the present study was to investigate whether LPS-induced septic shock could influence either the number, morphology or granular content of dura mater mast cells and to examine whether systemic administration of VIP and L-NAME could influence this response to LPS.

Methods

60 Sprague-Dawley rats of both sexes (200–250 g) were divided into four groups. Group 1: Controls ($n=20$), Group 2: Septics ($n=20$), Group 3: L-NAME ($n=10$), Group 4: VIP ($n=10$).

Rats were anaesthetized with urethane [1.5 g kg^{-1} intraperitoneal (i.p.)]. They were then infused with lipopolysaccharide from *Escherichia coli* serotype 0127:B8 Sigma L-3129, 25 mg kg^{-1} i.p. in sterile saline). L-NAME (Sigma N-5751, 30 mg kg^{-1} i.p.) and VIP (Sigma V-3628, 25 ng kg^{-1} i.p.) were injected 30 min after LPS administration. Control rats were infused with the same volume of sterile saline. Six hours later rats were deeply anaesthetized with urethane and the ascending aorta was catheterized for perfusion fixation. Rats were perfused with 200 ml cold heparinized phosphate buffer saline (PBS pH:7.4 0.1 M), followed by 500 ml of cold 4% paraformaldehyde in PBS solution at a pressure of 140 mmHg. After decapitation, the skull was opened carefully, the supratentorial dura mater was removed and was then post-fixed overnight by immersion in the same fixative solution.

All histochemical procedures were performed on free-floating and whole mount preparations.

Rat mast cell proteases (RMCP) immunolabelling

Following post-fixation, dura mater samples were rinsed in PBS six times for 10 min, and incubated in PBS+ (0.2% tritonX100+2.5% bovine serum albumin in PBS) for 1 h at room temperature, then incubated in polyclonal anti-RMCP1 antibody (Moredum Scientific MS-RMS10 developed in rabbit, diluted 1/1000 in PBS+) for 24 h at 4°C. After rinsing three times in PBS for 10 min, they were incubated in the secondary antibody (Swine anti-rabbit IgG FITC conjugate, DAKO F0054, diluted 1/200 in PBS+) for 1 h at room temperature and rinsed three times in PBS for 10 min, followed by a 3 h incubation in monoclonal anti-RMCP2 antibody (Moredum Scientific MS-RM2 developed in mouse, diluted 1/500 in PBS+). After rinsing in PBS, samples were incubated for 45 min with anti-mouse R-phycoerythrine (DAKO R0439, diluted 1/200 in PBS+). They were rinsed in PBS, the whole mounts were dried and mounted in Vectashield (Vector, H 1000).

Heparin and serotonin immunolabelling

Post-fixed dura maters were rinsed in PBS six times for 10 min, and incubated in PBS+ for 1 h at room temperature, then incubated in primary antibody (polyclonal anti-serotonin developed in rabbit, Sigma S 5545, diluted 1/5000 in PBS+) for 24 h at 4°C. They were rinsed three times in PBS for 10 min, incubated in the secondary antibody (Anti-Rabbit IgG TRITC conjugate, Sigma T 6778, diluted 1/400 in PBS+) for 2 h at room temperature and rinsed three times in PBS for 10 min. After anti-serotonin immunochemistry, mast cell heparin granule content was revealed by incubating the same samples in an avidin-FITC solution (ExtrAvidin-FITC conjugate, Sigma E 2761, diluted 1/400 in PBS+) for 2 h at room temperature. After rinsing three times in PBS for 10 min, the whole mounts were dried and mounted in Vectashield (Vector, H 1000).

Preparations were examined under a Leitz DMRX fluorescence microscope equipped with a cooled CCD video camera (Photonic Science) and with a computer-controlled motorized stage allowing optical tomography (Prior) for acquisition of digitized images. This made it possible to study labelled elements within the whole depth of the dura (~ 50 μm) and when necessary to obtain highly contrasted images even of weakly fluorescent elements by successive integration. Mast cells were counted on eight camera fields (total surface area = 3.52 mm^2) by using a X10 objective. Four areas contained the main branches of the middle meningeal artery and four did not contain major blood vessels in each dura. Immunoreactive (IR) and avidin-positive (heparin+) mast cells were counted in each area and these counts were summed.

Following removal, dura mater samples were rapidly frozen in liquid nitrogen and kept until biochemical assays. Frozen specimens were disrupted in 300 μl of ethanol/acetone (50 50^{-1}; v v^{-1}). After centrifugation, two 150 μl aliquots were evaporated under nitrogen and the dry residues were used for histamine and serotonin measurement. Radioenzymatic assay was used for histamine content determination, as described previously (Haimart *et al*., 1985). Serotonin content was measured using high-performance liquid chromatography and fluorometric detection (Kema *et al*., 1993).

Results are presented as mean $\pm$ s.d. with n equal to the number of animals. The statistical significance of the differences in mast cell granule heparin and serotonin content within different groups of rats was analysed by ANOVA followed by Bonferroni's t-test. The Student t-test was used to determine the significance of the differences in dura serotonin and histamine concentrations as well as in mast cell protease contents. $P<0.05$ was taken as significant.

Results

The number of mast cells containing RMCP

Double immunohistochemical labelling of rat dura mast cells revealed in individual cells the presence of granules containing either RMCP1 or RMCP2 or both. These three types of mast cells were present in different proportions and had various morphologies and locations. Dura of control rats exhibited 95% RMCP1-IR, 3% RMCP2-IR and 2% RMCP1/2-IR (Table 1). RMCP1-IR mast cells were mostly fusiform and were distributed at random in the tissue. Their mean shortest radius was 11.68 $\pm$ 1.75 μm. RMCP2-IR mast cells were round and mostly perivascular (Figures 1a and 2b). Their mean radius was significantly smaller than that of RMCP1-IR mast cells (8.34 $\pm$ 1.7 μm) (Figure 2a and b).

LPS administration did not cause any significant change in the total number of mast cells. The proportion of RMCP1-IR mast cells slightly but not significantly decreased to 69% of the total number, but the proportions of RMCP2-IR and RMCP1/2-IR mast cells significantly increased to 21 and 10% respectively (Table 1 and Figure 1b).

Table 1 Number of mast cells containing RMCP1, RMCP2 or both proteases

	RMCP1	*RMCP2*	*Both*	*Total*	n
Controls	300 $\pm$ 32.7	8.9 $\pm$ 7.5	4.1 $\pm$ 1.7	313 $\pm$ 26	4
Septics	241 $\pm$ 74	72 $\pm$ 21.8*	34.6 $\pm$ 21.3*	348.8 $\pm$ 67	4

Septic rats received LPS (25 mg kg^{-1} i.p.). Tissue samples were removed 6 h after LPS administration. Data presented as mean $\pm$ s.d. *Significantly different from the control animals.

The number of mast cells containing heparin and serotonin

Immunohistochemial analysis of mast cell granular heparin and serotonin content showed the presence of three populations of mast cells which contained either only serotonin, only heparin, or both molecules in all experimental groups. These three types of mast cells were present in different proportions and had various morphologies and locations. Dura of control rats exhibited 5% serotonin-IR, 4% heparin+ and 91% serotonin-IR/heparin+ (Figures 1c and 3). Serotonin-IR/heparin+ mast cells were mostly fusiform and were distributed at random in the tissue like RMCP1-IR mast cells. Serotonin-IR and heparin+ mast cells were round and mostly perivascular. The mean radius of heparin+ mast cells was 11.13 $\pm$ 1.5 μm. The mean radius of serotonin-IR mast cells was significantly smaller than that of heparin+ mast cells (8.58 $\pm$ 2.1 μm) (Figure 2c, d and e).

There was no significant difference in the total mast cell numbers among the experimental groups compared to controls (Figure 4). However, after LPS administration, the proportions of heparin+ and serotonin-IR mast cells slightly increased to 11 and 9% respectively, but these increases were not significant as compared to controls. VIP treatment of septic rats significantly increased both the number of serotonin-IR (14%) and heparin+ mast cells (14%) when compared to controls but not to septics (Figures 1d and 3). However, the number of serotonin-IR/heparin+ mast cells significantly decreased in septic and VIP-treated septic rats (Figure 3). L-NAME administration to septic rats did not lead to any significant change among the number of serotonin-IR, heparin+, and serotonin-IR/heparin+ mast cells as compared to other groups (Figure 3 and 4).

Dura mater serotonin and histamine content

LPS administration induced a significant decrease in serotonin concentration (from 13.2 $\pm$ 0.71 to 7.76 $\pm$ 2.12 pmol mg^{-1} fresh tissue) and a significant increase in histamine concentration (from 89.65 $\pm$ 4.9 to 116.9 $\pm$ 21.75 pmol mg^{-1} fresh tissue) (Table 2).

Discussion

The contribution of dura mater mast cells to sterile neurogenic inflammation has been well documented (Artico *et al*., 1998; Bergerot *et al*., 2000; Dimitriadou *et al*., 1990; Moskowitz & Macfarlane, 1993). However, to our knowledge there has been only one study related to behaviour of dura mater mast cells in nematode infection (Dimitriadou *et al*., 1997) and none related to septic inflammation. The present study is thus the first report concerning dura mater mast cells of septic rats treated or not with VIP and L-NAME.

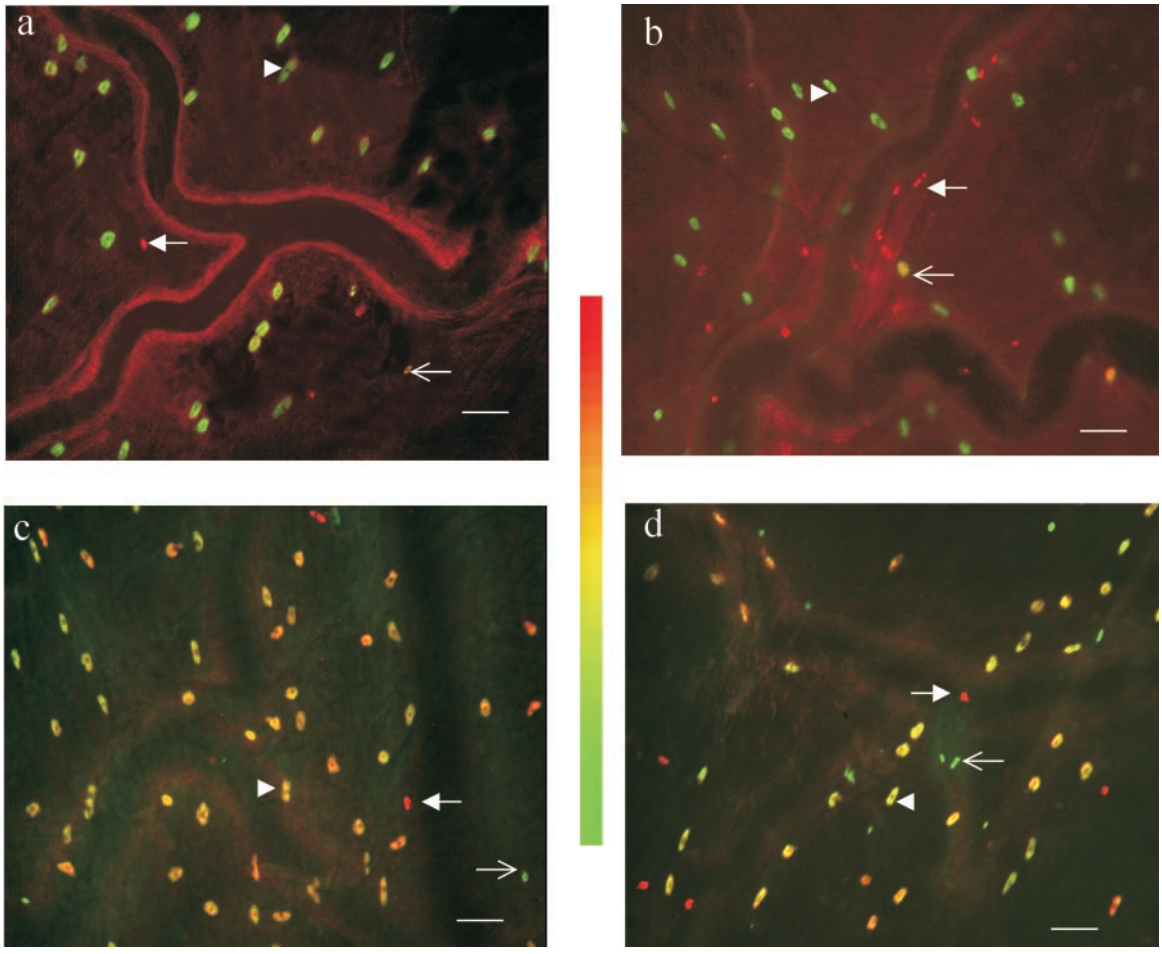

Figure 1 Typical appearance of mast cells in dura mater in control (a, c) and LPS-treated rats (b, d). Photographs are images merged from individual 256 gray level images corresponding respectively to FITC and TRITC fluorescences and previously transformed into false colours. A colour scale corresponding to these transformations is given. (a, b) RMCP immunohistochemistry (RMCP1-IR: green, RMCP2-IR: red). Note in (b) the greater proportion of RMCP2-IR mast cells (red cells, close arrow) and of RMCP1/RMCP2-IR mast cells (orange-yellow cells, open arrow). However, classical connective tissue type mast cells (CTMC) containing only RMCP1 (green cells, arrowhead) remain more numerous in LPS-treated rats. (c, d) Anti-serotonin immunohistochemistry (red) and visualization of heparin by avidin histochemistry (green). Note in (d) the greater proportion of serotonin-IR mast cells (red cells, close arrow) and of heparin+ mast cells (green cells, open arrow). However, classical CTMC containing both serotonin and heparin (orange-yellow cells, arrowhead) remain more numerous in LPS-treated rats. Scale bars = 100 μm.

Since their discovery it has been known that mast cells do not represent a homogeneous population, although all types share several characteristics. Evidence for histochemical and functional heterogeneity of mast cells was first given a sound basis in the 1960s by Enerback (1966). It was then well defined by many authors (see Metcalfe *et al*., 1997 for review). The classification of rodent mast cell subtypes is based on phenotypical and functional differences between connective tissue type mast cells (CTMC) and mucosal type mast cells (MMC). The most prominent phenotypical differences concern the cell shape, their histamine and proteoglycan content and their neutral protease synthesis. Dura mater mast cells have long been described as being a homogeneous CTMC population. Recently, Dimitriadou *et al*., using two specfic proteases, RMCPI and RMCPII as marker in rat dura mater, were able to identify three distinct populations of mast cells as being MMC (RMCP1-IR), CTMC (RMCP2-IR) and an intermediate phenotype (RMCP1/RMCP2-IR). They proposed that the presence of the intermediate RMCP1/RMCP2-IR population could correspond to a stage of differentiation of RMCP2-IR into RMCP1-IR cells, or to a reverse transition (Dimitriadou *et al*., 1997). Our results confirm the presence of these three types of mast cells in control rat dura mater, although we found a slightly different proportion for each type. Dimitriadou *et al*. found 91.8% RMCP1-IR, 8% RMCP2-IR, and 0.2% RMCP1/RMCP2-IR mast cells. In control rats, we observed 95% RMCP1-IR, 3% RMCP2-IR, and 2% RMCP1/RMCP2-IR mast cells. Interestingly the respective proportions of cells which are serotonin-IR/heparin+, serotonin-IR, and heparin+ were roughly similar. It is well known that heparin proteoglycan is only present in the granules of CTMC but not in MMC. Two amines, histamine and serotonin, are present in the granules of both CTMC and MMC of rodents (Dimlich *et al*., 1991). Thus, the present results show that serotonin-IR/heparin+ and only heparin+ mast cells are probably CMTC. Their proportion of 95% of the

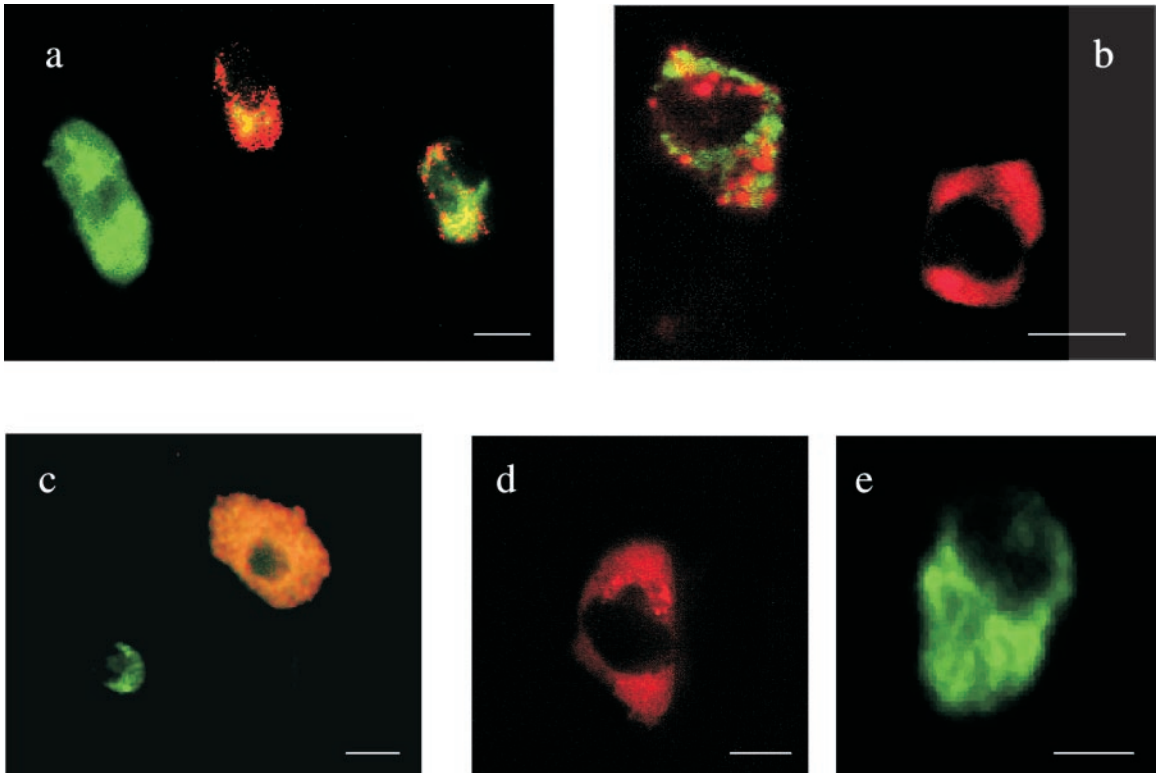

Figure 2 Mast cells at high magnification (laser-scanning confocal microscopy). (a, b) RMCP-1R mast cells. Note in (a) the presence of a mature RMCP1-IR cell (classical CTMC, green) neighbouring with two RMCP1/RMCP2-IR cells containing various proportions of both proteases. Granule location of proteases is clearly visible. In (b), a cell containing roughly equal amounts of RMCP1 and RMCP2 (green and red granules, respectively) is visible close to a pure RMCP2-IR cell. In the latter, the granule location of the protease is less obvious. (c, d and e) Serotonin-IR and heparin+ mast cells. Note in (c), the presence of serotonin-IR/heparin+ mast cell (classical CTMC, yellow) neighbouring with a small heparin+ mast cell. In (d), a mast cell containing only serotonin which resembles RMCP2-IR mast cell. In (e), heparin+ mast cell with higher magnification. Scale bars = 5 μm.

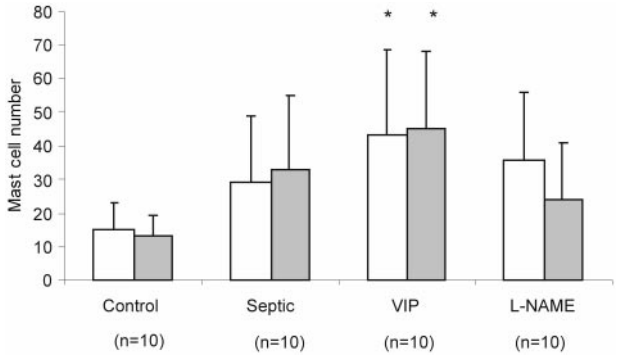

Figure 3 Number of serotonin-IR and heparin+ mast cells. Septic rats received LPS (25 mg kg^{-1} i.p.) L-NAME (30 mg kg^{-1} i.p.) and VIP (25 ng kg^{-1} i.p.) were infused 30 min after LPS administration. Tissue samples were removed 6 h after LPS administration. Blank columns represent serotonin-IR mast cells, gray columns represent heparin+ mast cells. Data presented as mean ± s.d. *Significantly different from control animals.

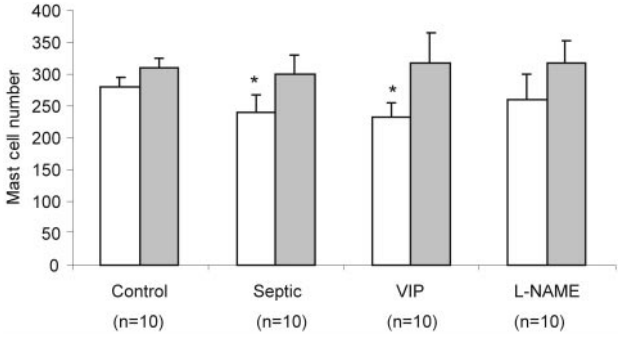

Figure 4 Number of serotonin-IR/heparin+ and total mast cells. Septic rats received LPS (25 mg kg^{-1} i.p.). L-NAME (30 mg kg^{-1} i.p.) and VIP (25 ng kg^{-1} i.p.) were infused 30 min later LPS administration. Tissue samples were removed 6 h after LPS administration. Blank columns represent serotonin-IR/heparin+ mast cells, gray columns represent total mast cells. Data presented as mean ± s.d. *Significantly different from control animals.

total mast cell population could be directly related to the 95% of RMCP1-IR mast cells. So while it is very likely that serotonin-IR/heparin+ mast cells are classical CTMC phenotype, granular cells containing only heparin could be a subtype of CTMC which does not synthesize serotonin or which releases serotonin in large amounts, the remaining cell concentration not being visible by indirect immunolabelling. Conversely, the populations of serotonin-IR mast cells which contain only serotonin and not visible heparin could be of the MMC-like phenotype. In fact, their size and proportion are comparable to those of RMCP2-IR mast cells.

Table 2 Dura mater concentration in serotonin and histamine (pmol mg^{-1} fresh tissue)

	Serotonin	*Histamine*	n
Controls	13.2±0.71	89.65±4.9	6
Septics	7.76±2.12*	116.9±21.75*	6

Septic rats received LPS (25 mg kg^{-1} i.p.). Tissue samples were removed 6 h after LPS administration. Data presented as mean±s.d. *Significantly different from control animals.

After LPS administration, the proportion of the three different mast cell populations changed. The number of RMCP1-IR mast cells (CTMC) decreased, although not significantly, and the respective numbers of RMCP2-IR (MMC) and RMCP1/RMCP2-IR (intermediate phenotype) mast cells significantly increased. It is well known that the population of MMC expands remarkably during T-cell dependent immune responses to certain intestinal parasites (Metcalfe *et al*., 1997). In addition, it has been shown that infection of the nematode *Nippostongylus brasiliensis* stimulates MMC proliferation in dura mater (Dimitriadou *et al*., 1997). The present study shows that systemic bacterial infection also stimulates RMCP2-IR mast cells (MMC) proliferation in dura mater. In the same way, LPS administration induced an increase in the proportion of heparin+ (proposed as a subtype of CTMC) and serotonin-IR mast cells (MMC-like) from 5 to 9% and from 4 to 11%, respectively, although these increases were not statistically significant. However, it significantly decreased the proportion of serotonin-IR/heparin+ mast cells (classical CTMC) from 91 to 80%, without any change in the total number of dura mater mast cells. It is possible that CTMC respond to sepsis by stopping their serotonin synthesis and/or by releasing it selectively to various extents. The fact that LPS also significantly increases dura histamine content without changing the total number of mast cells suggests that LPS induces a change in amine synthesis rather than a selective exocytosis. A decrease in synthesis or an increase in serotonin release in CTMC would explain the significant decrease in the dura serotonin content in septic rats. However, the number of cells containing only serotonin slightly increased after LPS administration. This apparent discrepancy could be due to the fact that the serotonin content of MMC (<0.5 pg $cell^{-1}$) is very small compared to the CTMC serotonin content (1–2 pg $cell^{-1}$) (Metcalfe *et al*., 1997). Therefore, the global decrease dura serotonin content could mainly reflect the general decrease in the CTMC serotonin content.

Since in both control and septic rats the shape and the respective proportions of the various types of mast cells were found to be similar when examining either their protease contents or their serotonin and heparin contents, we only analysed the latter to study of the effects of L-NAME and VIP. Indeed, we assumed that serotonin-IR/heparin+, only heparin+ mast cells were to be classified as CTMC, and only serotonin-IR mast cells as MMC, as discussed above.

It is well known that LPS administration induces NO synthesis. NO is a short-lived, fast-acting molecule formed from L-arginine by the enzyme NOS (Brown *et al*., 1998). L-NAME inhibits NOS non-selectively. There is a growing body of evidence to suggest that NO inhibits platelet–platelet and neutrophil–neutrophil interactions *in vitro* and *in vivo*. Using intravitral microscopy to study leucocyte behaviour in single post-capillary venules, Kubes *et al*., demonstrated that superoxide anion and mast cell activation were responsible for the onset of leukocyte adhesion associated with NO synthesis inhibition (Kubes *et al*., 1993). Inhibition of NO synthesis leads to increased levels of oxidants, mast cell activation and neutrophil adhesion, which are key features of a variety of inflammatory conditions. It has been proposed that NO may function as an anti-oxidant during the inflammation. It can reduce the primary source of oxidants by inhibiting neutrophil infiltration, inhibit the function of NADPH oxidase, the superoxide generating system found primarily in phagocytes, and inactivate superoxide directly (Mannaioni *et al*., 1991; Kubes *et al*., 1993; Kubes & McCafferty, 2000). Therefore, NO behaves as a physiologic barrier to superoxide anion. In the absence of nitric oxide, superoxide anion can activate various inflammatory cells including mast cells. There is increasing evidence that NO inhibits mast cell degranulation and histamine release and that NO synthesis inhibitors induce degranulation (Gaboury *et al*., 1996; Mannaioni *et al*., 1991). Tuncel *et al*. showed that during sepsis, L-NAME increases mast cell degranulation and significantly changes mast cell granular content in kidney. In this organ, the number of heparin-containing mast cells decreased and the number of mast cells with low-heparin concentration increased (Tuncel *et al*., 2000). However, the same authors did not find similar changes in liver under the same experimental conditions. The present study strongly confirms that L-NAME administration during sepsis does not lead to uniform reactions of mast cells in different organs. In fact, our observations of dura mast cells can be directly related to what was observed in the liver by Tuncel *et al*. (2000). In the present study, although L-NAME administration to septic rats did not cause significant change in the proportion of mast cells when compared to controls and septics, L-NAME administration to septic rats slightly increased the proportion of serotonin-IR mast cells from 9 to 11% and slightly decreased heparin+ mast cells from 11 to 7% of the total number when compared to septics. It is possible that these differences could be due to different concentrations of NO in the organs examined (Tuncel *et al*., 2001).

VIP is a potent protective neuropeptide against septic shock (Said, 1996; Tuncel *et al*., 2000). VIP increases the survival rate of septic animals (Delgado *et al*., 1999; Tuncel & Tore, 1998). Inhibition of NO synthesis failed to improve survival rate of septic rats (Tuncel & Tore, 1998). Among the many effects of VIP and L-NAME during sepsis, the most prominent was observed in mast cells in liver and kidney of rats (Tuncel *et al*., 2000). These authors concluded that mast cells, according to their location, display functional heterogeneity to septic insults and that VIP and to a lesser extent L-NAME have different effects on mast cells according to whether they are in liver or kidney. For example, VIP increased the number of mast cells with low heparin concentration in the liver but not in the kidney. The present results show that VIP significantly changes the proportion of the three populations of dura mater mast cells in septic rats as compared to controls, but not to septics, without changing the total number of mast cells. In other words, VIP decreases the proportion of classical CTMC (serotonin-IR/heparin+) from 91 to 72% while it increases the proportion of the two

other types [serotonin-IR (MMC-like) from 5 to 14% and heparin+ (CTMC subtype) from 4 to 14%]. Various *in vivo* studies have shown that the dose of 25 ng kg^{-1} VIP which was used in this study effectively inhibits degranulation of mast cells induced by LPS, cold-restraint stress, haemorrhagic shock and ischemia-reperfusion injury (Tikiz *et al*., 1991; 1992; Tuncel *et al*., 1996; 1998; Erden *et al*., 1998). However these authors did not observe any mast cell proliferation induced by VIP. On the contrary, our results strongly suggest that VIP increases the effects of sepsis on recruitment of immature mast cells (small round mast cells, containing no heparin and defined as MMC), since their increase in number become significant compared to controls. Thus, whatever the role(s) of the changes observed in dura mast cell phenotypes and content during sepsis, VIP but not NO appears to favour this (or these) role(s). This observation is reminiscent of that from Tuncel *et al*. in the liver but not in the kidney since they found an increased number of cells with low heparin concentration in this organ under the similar experimental conditions (Tuncel *et al*., 2000). However, the staining techniques used by these authors did not allow them to determine whether these cells were either MMC or CTMC, having lost their heparin. Furthermore, it is more difficult to obtain a reliable estimation in the whole organ of the relative proportions of various cell types from some sections of organs like kidney or liver than from whole mounted organs like dura mater.

In conclusion, the present study shows that the proportions of subpopulations of mast cells in rat dura mater are changed by septic insults. This may be a defense mechanism since mast cells are responsible for immediate hypersensitivity in immune reactions. If the dura mater play some 'lymphoid' role as a first immune barrier protecting the brain, the incidence of immune cells in this organ could be of great importance for brain integrity during sepsis, since their primary function is probably to protect the organism from endotoxic insults. It is also well known that mast cells interact with autonomic and sensory nerves during inflammation. In this way, they may initiate CNS responses to septic insults and mediate a part of this response through autonomic input.

This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS, UMR 5017), University Bordeaux II and University Osmangazi.

References

ARTICO, M., DE SANTIS, S. & CAVALLOTTI. C. (1998). Cerebral dura mater and cephalalgia: relationships between mast cells and catecholaminergic nerve fibers in the rat. *Cephalalgia,* **18,** 183–191.

BERGEROT, A., REYNIER-REBUFFEL, A.M., CALLEBERT, J. & AUBINEAU, P. (2000). Long-term superior cervical sympathectomy induces mast cell hyperplasia and increases histamine and serotonin content in the rat dura mater. *Neurosci.,* **96,** 205–213.

BRANDZAEG, P., OKTADALEN, O., KIERULF, P. & OPSTAD, P.K. (1989). Elevated VIP and endotoxin plasma levels in human gram-negative septic shock. *Regul. Pept.,* **24,** 37–44.

BROWN, J.F., CHAFEE, K.A. & TEPPERMAN, B.L. (1998). Role of mast cells, neutrophils and nitric oxide in endotoxin-induced damage to the neonatal rat colon. *Br. J. Pharmacol.,* **123,** 31–38.

DELGADO, M., MARTINEZ, C., POZO, D., CALVO, J.R., LECETA, J., GANEA, D. & GOMARIZ, R.P. (1999). Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) protect mice from lethal endotoxemia through the inhibition of TNF-alpha and IL-6. *J. Immunol.,* **162,** 1200–1205.

DIMITRIADOU, V., HENRY, P., BROCHET, B. & AUBINEAU, P. (1990). Cluster headache; ultrastructural evidence for mast cell degranulation and interaction with nerve fibres in human temporal artery. *Cephalalgia,* **10,** 221–228.

DIMITRIADOU, V., ROULEAU, A., TRUNG TUONG, M.D., NEWLANDS, G.J.F., MILLER, H.R.P., LUFFAU, G., SCHWARTZ, J.C. & GARBARG, M. (1997). Functional relationships between sensory nerve fibers and mast cells of dura mater in normal and inflammatory conditions. *Neurosci.,* **77,** 829–839.

DIMLICH, R.V.W., KELLER, J.T., STRAUSS, T.A. & FRITTS, M.J. (1991). Linear arrays of homogenous mast cells in the dura mater of the rat. *J. Neurocytol.,* **20,** 485–503.

ECHTENACHER, B., MANNEL, D.N. & HULTER, L. (1996). Critical protective role of mast cells in a model of acute septic peritonitis. *Nature,* **381,** 75–77.

ENERBACK, L. (1996). Mast cells in rat gastrointestinal mucosa. 1. Effects of fixation. *Acta Pathol. Microbiol. Scand.,* **66,** 289–302.

ERDEN, S., TUNCEL, N., AYDIN, Y., SAHINTURK, V., KOSAR, M. & TUNCEL, M. (1998). The effect of vasoactive intestinal peptide (VIP) and inhibition of nitric oxide on renal tissue injury of rats exposed to haemorrhagic ischemia and retransfusion: a possible interaction mechanism among mast cells and tissue histamine. *Ann. NY Acad. Sci.,* **865,** 570–581.

GABOURY, J.P., NIU, X. & KUBES, P. (1996). Nitric oxide inhibits numerous features of mast cell-induced inflammation. *Circulation,* **93,** 318–326.

GALLI, J.S. (1993). New concepts about the mast cell. *N. Engl. J. Med.,* **328,** 257–265.

HAIMART, M., LAUNAY, J.M., ZURCHER, G., CAUET, N., DREUX, C. & DAPARADA, M. (1985). Simultaneous determination of histamine and N-alpha-methylhistamine in biological samples by an improved enzymatic single isotope assay. *Agents Actions,* **16,** 71–75.

KEMA, I.P., SCHELLINGS, A.M., HOPPENBROUWERS, C.J., RUTGERS, H.M., DE VRIES, E.G.E. & MUKIET, F.A.J. (1993). High performance liquid chromatographic profiling of tryptophan and related indoles in body fluids and tissue of carcinoid patients. *Clin. Chim. Acta,* **221,** 143–158.

KUBES, P. & MCCAFFERTY, D.M. (2000). Nitric oxide and intestinal inflammation. *Am. J. Med.,* **109,** 150–158.

KUBES, P., KANWAR, S., NIU, X. & GABOURY, J.P. (1993). Nitric oxide synthesis inhibition induces leukocyte adhesion via superoxide and mast cells. *FASEB J.,* **7,** 1293–1299.

MANNAIONI, P.F., MASINI, E., PISTELLI, A., SALVEMINI, D. & VANE, J.R. (1991). Rat mast cells inhibit platelet aggregation by releasing a nitric oxide-like factor: influence histamine release. *Agents Action Suppl.,* **33,** 423–428.

MARSHALL, J.S. & BIENENSTOCK, J. (1994). The role of mast cells in inflammatory reactions of the airways, skin and intestine. *Curr. Opin. Immunol.,* **6,** 853–859.

METCALFE, D., BARAM, D. & MEKORI, Y. (1997). Mast Cells. *Physiol. Rev.,* **77,** 1033–1079.

MOSKOWITZ, M.A. & MACFARLANE, R. (1993). Neurovascular and molecular mechanisms in migraine headaches. *Cerebrovasc. Brain Metab. Rev.,* **15,** 533–538.

PARILLO, J.E. (1993). Pathogenetic mechanisms of septic shock. *N. Engl. J. Med.,* **328,** 1471–1477.

QUAN, N., STERN, E.L., WHITESIDE, M.B. & HERKENHAM, M. (1999). Induction of pro-inflammatory cytokine mRNAs in the brain after peripheral injection of subseptic doses of lipopolysaccharide in the rat. *J. Immunol.,* **93,** 72–80.

RAMOS, B.F., ZHANG, Y., ANGKACHATCHAI, V. & JAKSCHIK, B.A. (1992). Mast cell mediators regulate vascular permeability changes in Arthus reaction. *J. Pharmacol. Exp. Ther.,* **262,** 559–565.

REVHAUG, A., LYGREN, I., JENSSEN, G.T., GIERCKSKY, K.E. & BURHOL, P.G. (1988). Vasoactive intestinal peptide in sepsis and shock. *Ann. NY Acad. Sci.,* **527,** 536–545.

REYNIER-REBUFFEL, A.M., CALLEBERT, J., DIMIRIADOU, V., MATHIAU, P., LAUNAY, J.M., SEYLAZ, J. & AUBINEAU, P. (1992). Carbachol induces granular cell exocytosis and serotonin release in rabbit cerebral arteries. *Am. J. Physiol.,* **262,** R605–R611.

REYNIER-REBUFFEL, A.M., MATHIAU, P., CALLEBERT, J., DIMIRIADOU, V., FARJAUDON, N., KACEM, K., LAUNAY, J.M., SEYLAZ, J. & AUBINEAU, P. (1994). Substance P calcitonin gene-related peptide and capsaicin release in cerebrovascular mast cells. *Am. J. Physiol.,* **32,** R1421–R1429.

SAID, S.I. (1995). Vasoactive intestinal peptide. In *Airway smooth muscle, peptide receptors, ion channels, and signal transduction* ed. Raeburn, D. & Giembycz, M.A. pp. 87–113. Basel: Birkhauser Verlag.

SAID, S.I. (1996). Vasoactive intestinal peptide and nitric oxide: divergent roles in relation to tissue injury. *Ann. NY Acad. Sci.,* **805,** 379–387.

THEOHARIDES, T.C. (1996). The mast cells: a neuroimmunoendocrine master player. *Int. J. Tiss. Reac.,* **18,** 1–21.

TIKIZ, H., TUNCEL, N., AKIN, M.Z. & GURER, F. (1992). The effect of VIP and naloxone combination on survival rates in rats exposed to severe hemorrhage. *Peptides,* **13,** 83–90.

TIKIZ, H., TUNCEL, N., GURER, F. & BAYCU, C. (1991). Mast cell degranulation in hemorrhagic shock in rats and the effect of vasoactive intestinal peptide, aprotinin and H_1 and H_2-receptor blockers on degranulation. *Pharmacology,* **43,** 47–52.

TUNCEL, N. & TORE, F.C. (1998). The effect of vasoactive intestinal peptide (VIP) and inhibition of nitric oxide synthase on survival rate in rats exposed to endotoxin shock. *Ann. NY Acad. Sci.,* **865,** 586–589.

TUNCEL, M., DOGRUKOL-AK, D. & ERKASAP, N. (2001). Modified method for the determination of capillary electrophoresis nitric oxide-correlated nitrate in tissue homogenats. *J. Chromatogr. B.,* **751,** 251–263.

TUNCEL, N., ERKASAP, N., SAHINTURK, V., DOGRUKOL, AK, D. & TUNCEL, M. (1998). The protective effect of vasoactive intestinal peptide (VIP) against stress-induced gastric ulceration in rats. *Ann. NY Acad. Sci.,* **865,** 309–312.

TUNCEL, N., GURER, F., ARAL, E., UZUNER, K., AYDIN, Y. & BAYCU, C. (1996). The effect of vasoactive intestinal peptide (VIP) on mast cell invasion/degranulation in testicular interstitium of immobilized + cold stressed and β-endorphin-treated rats. *Peptides,* **17,** 817–824.

TUNCEL, N., TORE, F., SAHINTURK, V., AK, D. & TUNCEL, M. (2000). Vasoactive intestinal peptide inhibits degranulation and changes granular content of mast cells: a potential therapeutic strategy in controlling septic shock. *Peptides,* **21,** 81–89.

WILLIAMS, R.M., BIENENSTOCK, J. & STEAD, R.H. (1995). Mast cells: The neuroimmune connection. *Chem. Immunol.,* **61,** 208–235.

ZERVOS, E.E., KRAMER, A.A., SALHAB, K.F., NORMAN, J.G., CAREY, L.C. & ROSEMURGY, A.S. (1999). Sublethal hemorrhage blunts the inflammatory cytokine response to endotoxin in a rat model. *J. Trauma,* **46,** 145–149.

(Received April 23, 2001
Revised July 26, 2001
Accepted September 21, 2001)