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Ultrastructural evidence of brain mast cell activation without degranulation in monkey experimental allergic encephalomyelitis

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Abstract

Experimental allergic encephalomyelitis (EAE) is an animal model for the human demyelinating disease multiple sclerosis (MS). Increased permeability of the blood–brain barrier (BBB) precedes the development of clinical or pathologic findings in MS and may be induced by perivascular brain mast cells secreting vasoactive and proinflammatory molecules. Brain mast cells were investigated ultrastructurally in acute EAE of the non-human primate common marmoset *Callithrix jacchus*, which develops a mild neurologic relapsing–remitting course. Control diencephalic samples contained perivascular mast cells with mostly intact electron dense granules. In contrast, EAE samples had marked demyelination and mast cells with numerous altered secretory granules; their electron dense content varied in amount and texture with a "honeycomb" or "target" appearance, but without degranulation. These changes were evident even before the development of any clinical symptoms and suggest that brain mast cells may be involved in EAE, and possibly MS, through a unique process that may involve selective secretion of molecules able to disrupt the BBB.

Keywords: Blood–brain barrier; Experimental allergic encephalomyelitis; Mast cells; Secretion

1. Introduction

Experimental autoimmune or allergic encephalomyelitis (EAE) in animals has been used extensively as an animal model for multiple sclerosis (MS), the human demyelinating disease of the CNS (Raine, 1984; Smith and McDonald, 1999). In MS lesions, demyelination appears to be mediated by blood-borne mononuclear cells that penetrate the blood–brain barrier (BBB) at multiple sites (Raine, 1984; Chang et al., 2002; Paty and Arnold, 2002). This cellular infiltrate consists primarily of T cells and macrophages (Hauser et al., 1986). Most animal studies have used EAE in rodents, but these models developed severe neurologic symptoms with variable, but mild CNS demyelination. Instead, EAE of the non-human primate common marmoset, *Callithrix jacchus*, is characterized by a mild neurologic relapsing–remitting course with pathological features of primary demyelination, reactive gliosis and mononuclear cell infiltration more reminiscent of MS (Massacesi et al., 1995).

Increased BBB permeability is an important early event in MS and precedes any pathological or clinical findings (Kermode et al., 1990; Stone et al., 1995). Increasing evidence has led to the suggestion that brain mast cells may regulate permeability of the BBB and leukocyte infiltration into the brain (Theoharides, 1990). In fact, mast cell activation was shown to induce increased permeability of the BBB (Linthicum et al., 1982; Zhuang et al., 1996).

Mast cells are located perivascularly, often in close association to neurons (Dimitriadou et al., 1987, 1990; Roznieckl et al., 1999), and are critical for allergic (Galli, 1993) and neuroinflammatory reactions (Theoharides, 1996). Mast cells have been identified in autoimmune brain

Abbreviations: BBB, blood–brain barrier; CFA, complete Freund's adjuvant; CSF, cerebrospinal fluid; CTMC, connective tissue mast cell; EAE, experimental allergic encephalomyelitis; MMC, mucosal mast cells; MS, multiple sclerosis; MBP, myelin basic protein; NGF, nerve growth factor; RMCP, rat mast cell protease; SCF, stem cell factor; TNF, tumor necrosis factor.

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demyelination (Raine, 1984) in brain lesions of EAE (Orr, 1988; Bo et al., 1991) and of MS (Johnson et al., 1988; Olsson, 1974; Krüger et al., 1990; Toms et al., 1990; Ibrahim et al., 1996). Mast cells can be activated by myelin basic protein (MBP) (Johnson et al., 1988; Theoharides et al., 1991) leading to brain demyelination and myelin degradation in vitro (Dietsch and Hinrichs, 1991) that is enhanced by estradiol (Theoharides et al., 1993a,b). Evidence of in vivo mast cell activation was provided by increased cerebrospinal fluid (CSF) histamine in EAE (Tuomisto et al., 1983), and increased mast cell tryptase in CSF of MS patients (Roznieckí et al., 1995).

Mast cells secrete vasoactive molecules such as bradykinin, histamine, tumor necrosis factor (TNF), and nitric oxide (NO), all of which could increase BBB permeability. In fact, increased brain vascular permeability was shown in response to the mast cell secretogogue compound 48/80 in pigeons (Zhuang et al., 1996). More recently, acute restraint stress in rats was shown to increase BBB permeability to ^{99}Technetium gluceptate, a process inhibited by the "mast cell stabilizer" disodium cromoglycate (Esposito et al., 2001). Moreover, acute stress could not affect BBB permeability in W/W^{v} mast-cell-deficient mice (Esposito et al., 2002), in which the development of EAE was substantially delayed and attenuated (Secor et al., 2000). Finally, acute stress decreased the time required for EAE development in mice (Chandler et al., 2002), possibly explaining why acute stress leads to exacerbation of symptoms in remitting–relapsing MS (Warren et al., 1982; Goodin et al., 1999; Mei-Tal et al., 1970; Poser, 2000; Mohr et al., 2000). Such findings have led to the recent suggestion that mast cell may serve as a new target for MS therapy (Zappulla et al., 2002).

In the present study, brain mast cells were investigated in non-human primate EAE. The results indicate that mast cells were activated in areas of demyelination apparently through intragranular changes indicative of selective secretion of vasoactive and lytic mediators.

2. Methods

2.1. Induction of EAE

Common marmoset *C. jacchus* male monkeys ($N=5$) were kept under standard laboratory living conditions (Massacesi et al., 1995). Animals were maintained in primate colonies at the New England Regional Primate Research Center and were cared for in accordance with the guidelines of the Committee on Animals of the Harvard Medical School, and those of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Monkeys were immunized by four injections administered at one time to both shoulders (dorsal axilla) and hips (inguinal region) with 0.6 ml each containing 200-mg fresh-frozen postmortem human brain white matter homogenate emulsified in complete Freund's adjuvant (CFA; Difco Laboratories, West Molesay, Surrey, UK) with 32-μg (3 mg/ml) *Mycobacterium tuberculosis* (H37Ra strain from DIFCO). Animals were allowed food and water ad libitum.

On the day of immunization and again 2 days later, inactivated *Bordetella pertussis* organisms (*B. pertussis* vaccine, Massachusetts Public Health Department Biological Laboratories, Boston, MA) were diluted in 10 ml of saline solution and administered intravenously. In animals that developed fever due to the initial pertussis vaccine, the second pertussis injection was delayed by 1 to 2 days. Two to five weeks after immunization, some animals developed local skin ulcerations related to the CFA. In these animals, cephalexin (20 mg/kg twice daily for 10 days) was administered intramuscularly to prevent secondary infection.

EAE was assessed by clinical and pathological criteria. A standardized scoring system was employed to record the severity of clinical disease: 0 = normal neurological findings; 1 = lethargy, anorexia, weight loss; 2 = ataxia, and either paraparesis/monoparesis, sensory loss, gaze palsy, or blindness; 3 = paraplegia or hemiplegia; 4 = quadriplegia.

Animals were killed at different times after immunization as reported previously (Massacesi et al., 1995). The CNS was removed and part of it was fixed in 10% formalin for routine histology. Paraffin sections of brain and spinal cord were stained with hematoxylin and eosin, or with Luxol fast blue. For each coronal brain section or horizontal spinal cord section, the histopathological findings of inflammation and demyelination were each independently graded according to an arbitrary scale:

Inflammation score	
0	No inflammation present
1+	Rare (1–3) perivascular cuffs/average whole section
2+	Moderate numbers (3–10) of perivascular cuffs/section; may have meningeal inflammation
3+	Widespread perivascular cuffing and parenchymal infiltration by inflammatory cells
Demyelination score	
0	No demyelination present
1+	Rare (1–3 lesions/section) foci of demyelination
2+	Moderate (3–10 lesions/section) demyelination
3+	Extensive demyelination with large confluent lesions

The clinical severity as well as the degree of CNS demyelination and inflammation were scored by Dr. L. Massacesi (see Acknowledgments) who was blind to the treatment code and did not participate in the subsequent ultrastructural evaluation. The clinicopathological results of the animals used in this study were reported previously (Massacesi et al., 1995) and are summarized in Table 1. Of the six EAE monkeys studied, (a) one was killed at day 550 and had a clinical score of 0, (b) four were killed at disease onset and had a clinical score of 2–3, while (c) one was followed without intervention and was killed when it had a severe relapse.

Table 1
Clinicopathological characteristics of the monkeys with EAE studied

Animal no.	Time of death (days)	Clinical score	Demyelination	Inflammation
1	550	0	++	++
2	25	2	++	++
3	33	2	+++	+++
4	45	2	++	++
5	188	3	++	+++
6	7110	2, 4	+++	+++

Details on these monkeys were published previously (Massacesi et al., 1995).

2.2. Fixation for light microscopy

Brain and spinal cord samples were obtained within 1 h of death, usually on day 14 after immunization and were fixed with a mixture of formaldehyde: glacial acetic acid: methanol (FAM, 1:1:8) for another 30 min at room temperature. For histochemical examination, sections were cut 30-μm thick using a vibratome and stained with 0.25% toluidine blue, pH 2.5, for 45 min at room temperature (Theoharides et al., 1993a,b). All sections were evaluated at × 200, while some sections were photographed at × 400 using a Nikon inverted microscope (Don Santo, MA).

2.3. Ultrastructural analysis

For electron microscopy, tissue sections were fixed in modified Karnovsky's medium containing 2% paraformaldehyde, 3% glutaraldehyde and 0.1% tannic acid in 0.1 M phosphate buffer (pH 7.4) and processed as before (Dimitriadou et al., 1990). Each diencephalon was cut in about 50 blocks without retaining any orientation. Three non-sequential 0.5-μ sections were chosen from five random blocks of diencephalon, known to be the richest brain area in mast cells (Pang et al., 1996). The maximum number of mast cells captured in each section rarely exceeded two as their diameter was 5–10 μ. Two photographs were taken of any section containing mast cells at a magnification of about × 10,000. All granules within the section were counted and evaluated; pictures at higher magnification were also obtained to improve the resolution when necessary. Mast cell activation was determined by the degree of secretory granule alterations characterized by (a) partial content giving the appearance of a "target", (b) electroluscent granules containing fine "fibrillar" material, (c) "empty" granules, as well as (d) lipid bodies (Letourneau et al., 1996). Brain inflammation and demyelination was determined by: (a) perivascular cuffing, (b) parenchymal inflammatory cell infiltration, (c) presence of lipid-laden

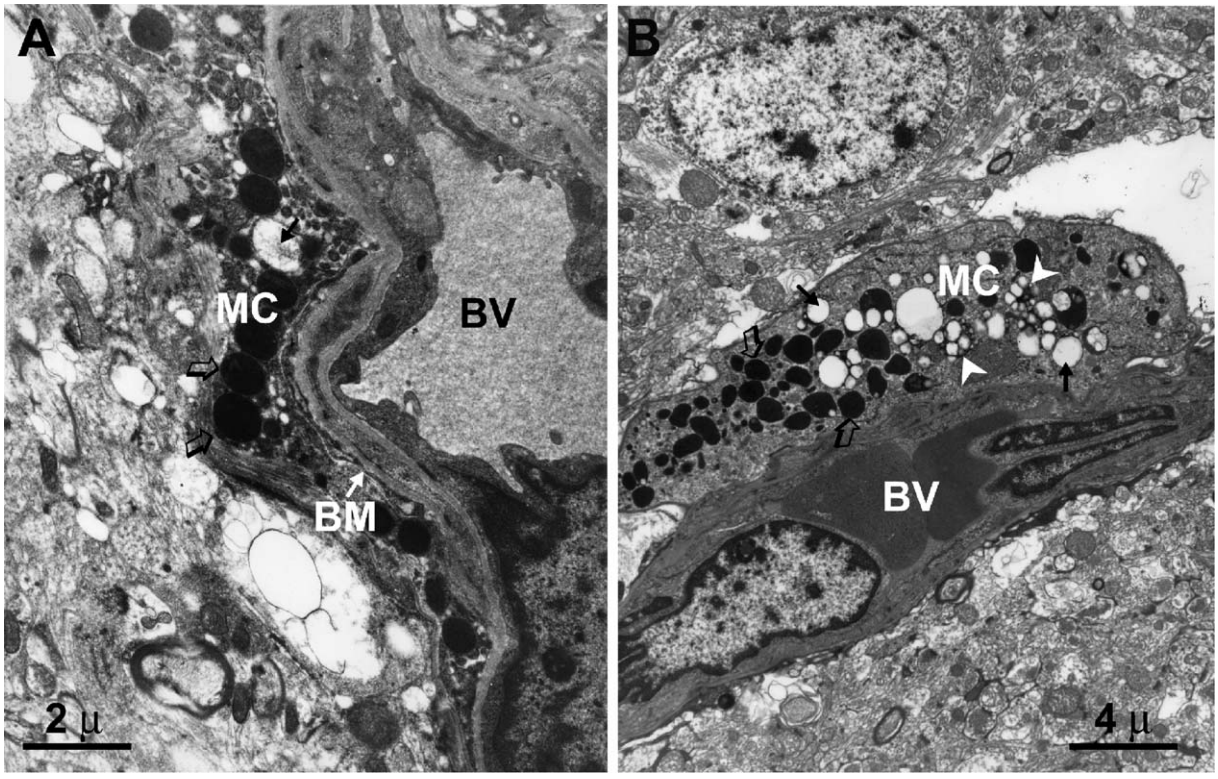

Fig. 1. Electron photomicrographs of two perivascular mast cells from the diencephalon of (A) a "control" animal without clinical symptoms of EAE with mostly intact electron dense granules (open arrow) and (B) an animal with EAE characterized by few intact (open arrows), numerous empty (solid arrows) and some secretory granules containing altered electron dense content (white arrow heads). BM = basement membrane; BV = blood vessel; MC = mast cell. Scale bars: A = 2 μ; B = 4 μ.

Table 2
Morphologic changes of diencephalic mast cell secretory granules indicative of activation

Conditions	Ultrastructural appearance of secretory granules (%total)			
	Normal	Altered	Empty	Activation (A + E)
Control[&] ($N_1 = 3$, $N_2 = 27$)	65.6 ± 14.2 ($n = 189$)	22.6 ± 9.2 ($n = 75$)	11.9 ± 12.1 ($n = 31$)	34.4
EAE ($N_1 = 5$, $N_2 = 88$)	30.9 ± 27.9 ($n = 138$)*	30.6 ± 19.6 ($n = 81$)[+]	37.9 ± 19.4 ($n = 155$)[#]	68.5*

N_1 = number of monkeys studied; N_2 = number of mast cells studied; n = number of granules studied; A = altered; E = empty; [&]includes one monkey that did not develop clinical symptoms of EAE.
*$p < 0.001$ compared to control.
[+] $p = 0.436$ compared to control.
[#] $p = 0.0058$ compared to control.

macrophages, fibrillary debris and (d) any reduction in myelin thickness in comparison to the size of the axon.

2.4. Statistical analysis

Analysis of the morphometric findings was carried out using the non-parametric Mann–Whitney U test; significance is denoted by $p < 0.05$.

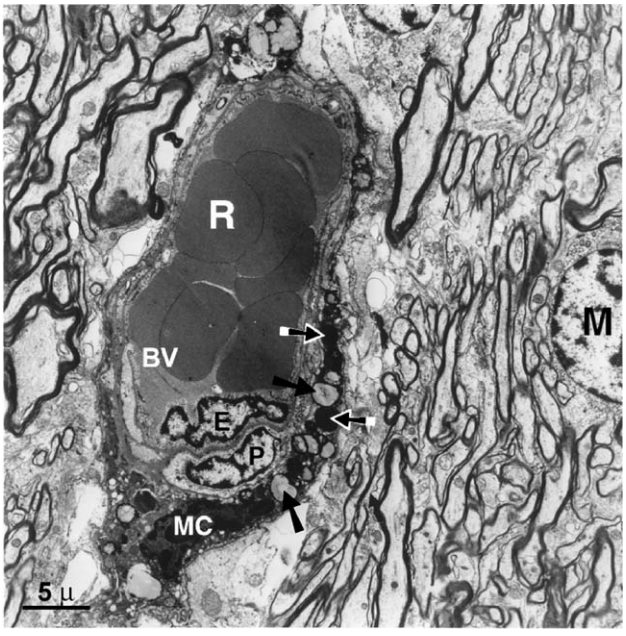

Fig. 2. Electron micrograph of a perivascular diencephalic mast cell from an animal with EAE. A venule full of red blood cells (R) is shown where the endothelial cell (E) is surrounded by a pericyte (P), which in turn is tightly embraced by a mast cell (MC). Note intragranular activation with loss of electron dense material in numerous mast cell secretory granules that appear empty, possibly containing lipid (solid arrow); some secretory granules are intact with homogeneous electron dense content (solid arrows against white background). Also note intense demyelination throughout the section. R = red blood cell; E = nucleus of endothelial cell; P = nucleus of pericyte; MC = nucleus of mast cell; M = nucleus of macrophage. Scale bar = 5 µ.

3. Results

The clinicopathological results of the animals used in this study were reported previously (Massacesi et al., 1995) and are summarized in Table 1. Of the six EAE monkeys studied, (a) one was killed at day 550 and had a clinical score of 0, (b) four were killed at disease onset and had a clinical score of 2–3, while (c) one was followed without intervention and was killed when it had a severe relapse. Clinical signs of EAE were present in all immunized monkeys ($N = 5$), except for the one mentioned above, at various days post immunization (Table 1). In the brain of

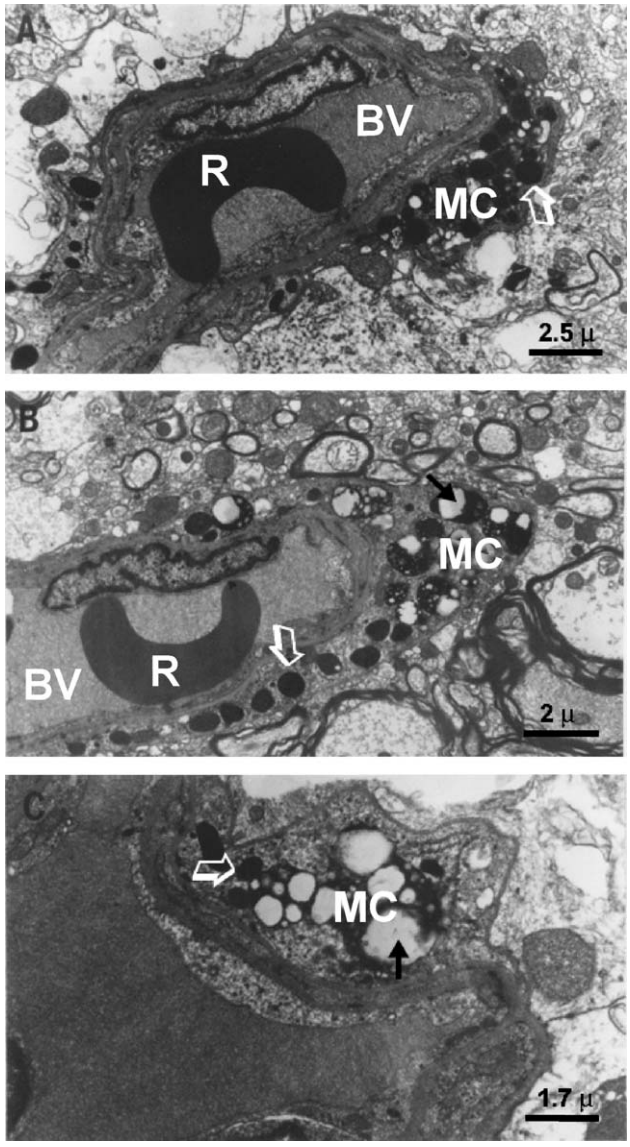

Fig. 3. Electron micrographs of perivascular diencephalic mast cells from animals with EAE. Note numerous altered secretory granules characterized by partial or total loss of electron dense material. (A) minimal activation; (B) activation of about half the granules shown at the right; (C) almost total activation with enlarged granules apparently containing lipid. Intact granules = open arrows, empty granules = solid arrows. BV = blood vessel; MC = mast cell; R = red blood cell. Scale bars: A = 2.5 µ; B = 2 µ; C = 1.7 µ.

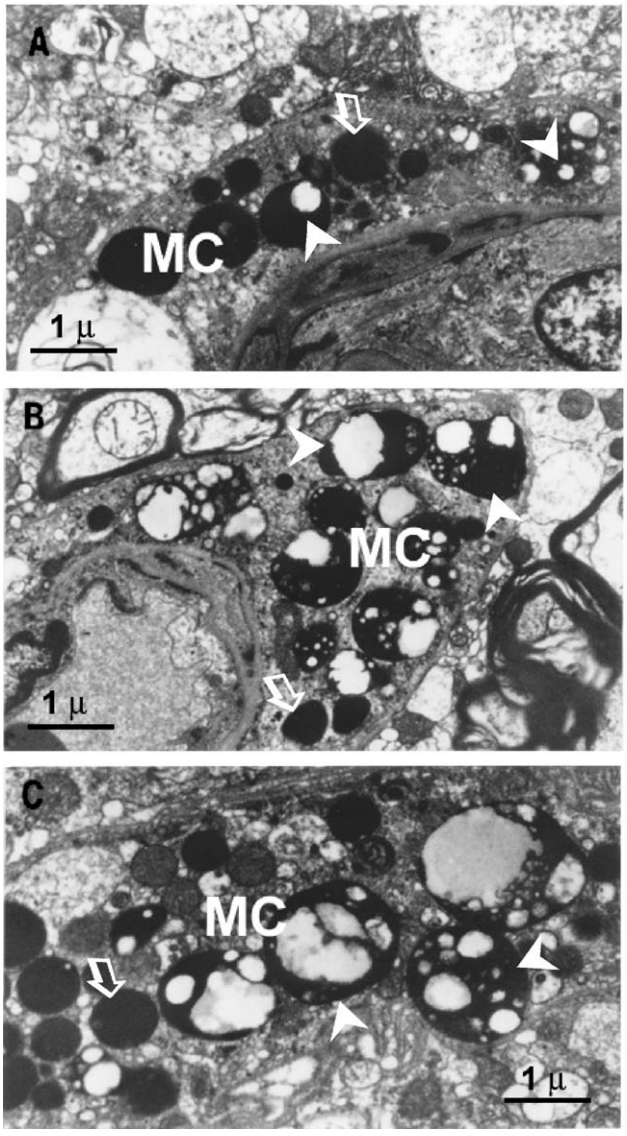

Fig. 4. Electron micrographs of activated diencephalic mast cells from EAE animals. Note: (A) Many secretory granules are intact (open arrow), while two have altered content (white arrow head); (B) almost all secretory granules have been altered with spot-like or partial loss of electron dense content (white arrow head); (C) altered granules appear clearly swollen with distinct loss of electron dense content giving the appearance of "punched-out" areas (white arrow head). Scale bars: A, B and C = 1 µ.

EAE animals, there were multiple acute perivascular lesions of primary demyelination with infiltrating macrophages, mostly in white matter; some were also present at white/gray matter junctions and in the diencephalon (specific results not shown).

We investigated the number and morphology of mast cells in the diencephalon because it is the richest brain area in mast cells. Light microscopic observations of sections stained with acidified toluidine blue identified metachromatic cells that were almost entirely perivascular and increased in animals with EAE (results not shown). Ultrastructural observations of control animals ($N_1 = 2$), as well as the one that was immunized but did not develop EAE ($N_1 = 1$), indicated that mast cells in the diencephalon were strictly perivascular and their secretory granules appeared mostly intact with homogeneous electron dense content (Fig. 1A). Diencephalic mast cells in control monkeys had about 66% of their secretory granules intact (Table 2). Of the rest, about 24% contained intragranular alterations, while 10% looked empty (Table 2).

In contrast, diencephalic areas from EAE animals with clinical symptoms contained perivascular mast cells in which the majority of the granules had altered content and texture of their electron dense granules (Fig. 1B). A representative section from one acute EAE diencephalic lesion is shown in Fig. 2, where intense perivascular demyelination with one macrophage is noted. The blood vessel lumen is congested with erythrocytes, while the endothelial cell is surrounded by a pericyte, which in turn is embraced by a mast cell (Fig. 2). The cytoplasm of this mast cell contains some intact, electron dense secretory granules, but also many granules that are almost devoid of electron dense material and appear empty, although they may contain lipid (Fig. 2).

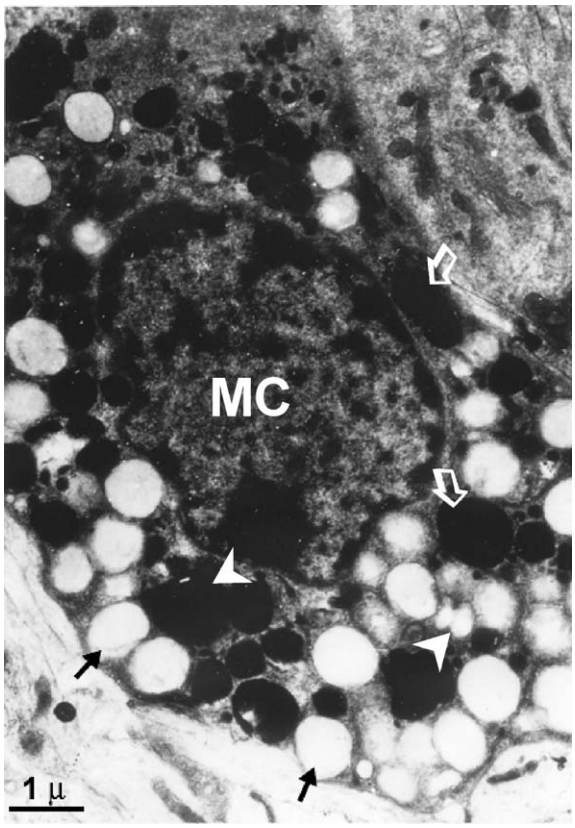

Fig. 5. Higher magnification of a diencephalic mast cell from an EAE animal containing some intact secretory granules (open arrows), but many characteristically "empty" secretory granules without any electron dense material (solid arrows), but without any evidence of typical exocytosis; a few granules have evidence of altered content and texture of their electron dense content (whited arrow heads). Scale bar = 1 µ.

The ultrastructure of mast cells from EAE animals showed that most granules had obvious intragranular changes consisting of either partial or complete loss of electron dense material (Figs. 3 and 4). Many granules contained fine fibrillar material, while others had distinct changes in their electron dense substructure reminiscent of ''target'', ''punched-out'' or ''honeycomb'' appearance (Fig. 4). A number of mast cells contained electroluscent or ''empty'' granules that may contain lipid (Fig. 5). However, there was no evidence of degranulation by compound exocytosis and no apparent direct communication with the extracellular space.

In contrast to 66% of intact granules in control animals, only 37% of granules were intact in EAE animals; of the rest, 41% were empty and 23% had intragranular alterations (Table 1). The difference between the total activated mast cells in EAE (68.5%) as compared to controls (34.4%) was highly significant ($p < 0.0058$).

4. Discussion

The method used in the present study to actively induce EAE in monkeys has been well established (Massacesi et al., 1995). This model, in contrast to rodents, is more like MS in that the brain of the affected monkeys is characterized by areas of intense demyelination (Massacesi et al., 1995). The present results show the majority of diencephalic mast cells are activated in monkeys with EAE, often in acute lesions of active demyelination. Mast cells had previously been noted in the brain of animals with EAE (Orr, 1988; Theoharides, 1990; Bo et al., 1991) and in MS (Johnson et al., 1988; Olsson, 1974; Krüger et al., 1990; Toms et al., 1990; Ibrahim et al., 1996). Some of these mast cells appeared to be activated at the light microscope level (Bo et al., 1991; Stanley et al., 1990; Brenner et al., 1994). The morphology of the brain mast cells in the present study is distinct from that of degranulation by compound exocytosis typical of connective tissue mast cells (CTMC); instead, the ultrastructure of the brain mast cell secretory granules in EAE is characterized by variable amounts and texture of electron dense material without exocytosis, as previously noted in rodents (Dimitriadou et al., 1990). The hallmark of brain mast cells from EAE animals is the ''punched-out'' secretory granules, as well as others that appear entirely empty, even though they may contain lipid. The unique morphology of activated brain mast cells was also noted in rodent EAE (Bo et al., 1991) and is reminiscent of the appearance of ''immature'' granules or mast cells that actively synthesize secretory granules (Dvorak et al., 1993). Activated intracranial mast cells were previously reported to contain lipid bodies (Ibrahim et al., 1979; Dimitriadou et al., 1990) that are considered to be important in phospholipid degradation (Dvorak et al., 1983). This type of mast cell activation is also similar to ''piece meal'' degranulation previously observed in the gastrointestinal tract (Dvorak et al., 1992) and bladder (Theoharides et al., 1995), especially in mast cells associated with nerve endings (Dvorak et al., 1992; Letourneau et al., 1996). Such activation could be associated with ''differential'' release (Theoharides et al., 1982) of mast cell secretory products (Kops et al., 1990), and was recently shown to involve selective release of IL-6 (Kandere-Grzybowska et al., in press). An almost identical appearance to the ''empty'' granules we describe herein was identified in intraparenchymal mast cells from affected cerebellar white matter in transgenic mice overexpressing IL-3 and was termed ''in site degranulation'' (Powell et al., 1999). Mast cell infiltration and activation at sites of brain demyelination in the thalamus and cerebellum appeared to accompany BBB defects documented with horseradish peroxidase leakage in brain parenchyma (Powell et al., 1999). Increased permeability of the BBB is now recognized as a key early event that precedes demyelination or clinical signs in MS, but the molecular basis of BBB alterations is unknown (Kermode et al., 1990; Moor et al., 1994; Stone et al., 1995; De Vreis et al., 1997).

Mast cells had been proposed as the ''immune gate'' to the brain (Theoharides, 1990) and have since been shown to increase BBB permeability in response to acute stress (Esposito et al., 2001, 2002). Such findings may be relevant to reports suggesting that acute stress may hasten EAE development (Chandler et al., 2002) and precipitate or worsen symptoms of relapsing–remitting MS (Warren et al., 1982; Goodin et al., 1999; Mei-Tal et al., 1970; Poser, 2000; Mohr et al., 2000). Mast cell-derived molecules (Theoharides, 1996) such as histamine (Olsen, 1987), arachidonic acid (Unterberg et al., 1987), bradykinin (Schurer et al., 1989) and serotonin (Sharma et al., 1990) have been shown to decrease BBB integrity (Abbott, 2000). Mast cells are also a rich source of cytokines including TNF-α (Galli, 1993) which induces endothelial leukocyte adhesion molecule-1 (Walsh et al., 1991). Brain mast cells were recently shown to release TNF-α upon immunologic stimulation (Cocchiara et al., 1998) and EAE could not develop in TNF-α knockout mice (Probert et al., 1997). Moreover, soluble TNF receptor (Hartung et al., 1995) and adhesion molecules (Tsukada et al., 1993; Sharief et al., 1993) were detected in CSF of MS patients and may contribute to BBB impairment (Rieckmann et al., 1993) and immune demyelination (Cannella et al., 1991; Archelos et al., 1999).

Mast cells derive from a distinct precursor cell in the bone marrow, enter the brain from the leptomeninges (Lambracht-Hall et al., 1990) and mature in the local microenvironment (Galli, 1993). During neonatal development in the rat, two brain mast cell populations can be distinguished (Dimitriadou et al., 1997): one with typical characteristics of CTMC containing mast cell protease (RMCP)-I and a second resembling mucosal mast cells (MMC) identified by its content of RMCP-II (Dimitriadou et al., 1996); a third type containing heterogenous secretory granules and lipid bodies has been termed neurolipomastoid (Ibrahim et al., 1979; Dimitriadou et al., 1990). Brain mast

cells of the adult rat are most abundant in the thalamus (Goldschmidt et al., 1984), the hypothalamus and median eminence (Dropp, 1976). Diencephalic (Edvinsson et al., 1977; Ibrahim et al., 1979; Olsson, 1968; Ibrahim, 1974; Orr, 1988) mast cells were definitively characterized and were shown to contain histamine, heparin, RMCP-I, as well as mRNA for immunoglobulin E (IgE) binding protein (FcεRI) (Pang et al., 1996); another paper also reported them to express FcεRI (Toms et al., 1990), but lack the c-kit receptor (Pang et al., 1996; Shanas et al., 1998). Mast cells can be activated by neurotransmitters (Dimitriadou et al., 1990), and by antidromic nerve stimulation (Dimitriadou et al., 1992) contributing to vasodilation (Dimitriadou et al., 1992); they can also be stimulated by myelin basic protein leading to demyelination in vitro (Johnson et al., 1988; Dietsch and Hinrichs, 1991; Theoharides et al., 1991, 1993a,b; Brenner et al., 1994).

Brain mast cell activation in EAE could be either causally related or an epiphenomenon. However, the former possibility is supported by the fact that such changes were present even in the animal that had not developed clinical signs by the time it was killed. Moreover, development of EAE was delayed and reduced in W/W^v mast-cell-deficient mice that had otherwise intact immune function (Secor et al., 2000). Intracisternal administration of the mast cell secretagogue compound 48/80 before immunization reduced the extent of EAE, presumably due to depletion of mast cell mediators (Stanley et al., 1990). In addition, the mixed serotonin and H_1-receptor antagonist cyproheptadine, which also inhibits mast cell secretion (Theoharides et al., 1985), reduced EAE (Dietsch and Hinrichs, 1989); inhibition of EAE was also achieved by the experimental mast cell "stabilizer" picroximil (Dietsch and Hinrichs, 1989).

EAE could also be reduced by the histamine-1 receptor antagonist hydroxyzine (Dimitriadou et al., 2000), which can also inhibit mast cell activation (Theoharides et al., 1985) and neurogenic inflammation (Minogiannis et al., 1998). Recently, a double blind placebo-controlled pilot clinical study showed that hydroxyzine could prevent worsening of disability in relapsing–remitting MS (Theoharides et al., 2002). The possible relevance of brain mast cells to MS is supported by the fact that the level of the specific human mast cell enzyme tryptase was increased in the CSF of MS patients (Rozniecki et al., 1995). Moreover, gene array of MS lesions showed overexpression of mast cell molecules (Lock et al., 2002) generating new hope for MS therapy (Tompkins and Miller, 2002). Mast cells may participate in the pathophysiology of EAE and MS primarily by increasing permeability of the BBB (Theoharides, 1990). Additional mast cell precursors recruited from the circulation may then enter the brain parenchyma and participate in local inflammation and/or demyelination Dines and Powell, 1997. The possibility of inhibiting brain mast cell activation may offer a new therapeutic approach (Zappulla et al., 2002) that could complement current treatment modalities which primarily address the function of the immune system.

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