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Early influx of macrophages determines susceptibility to experimental allergic encephalomyelitis in Dark Agouti (DA) rats

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ABSTRACT

Experimental allergic encephalomyelitis (EAE) is characterized by inflammatory infiltrates of myelin antigen(s) specific T cells and consecutive demyelination. Injection of encephalitogen into the footpads induces disease in genetically susceptible Dark Agouti rats (DA) but not in Albino Oxford (AO) rats although mild inflammatory infiltrates are observed in both strains early after disease induction. In addition, only DA rats develop disease when cells from (AO × DA) F_1 hybrids are passively transferred into sub-lethally radiated AO and DA parent hosts. The aim of the study was therefore to examine the participation of accessory cells, macrophages, dendritic cells and microglia in EAE development at the level of the target tissue in these two strains using specific membrane markers. We demonstrate here that in the induction phase of EAE in DA rats, macrophages ($CD68^+$; $CD45^{hi}CD11b^+$) are the first detectable infiltrating cells in the subpial regions of the spinal cord but were not found in AO rats. During the same period, resident microglial cells which are of the ramified variety are observed in both DA and AO rats. In DA rats at the peak of disease, when profuse influx of T cells is seen, macrophages and dendritic cells appear in the parenchyma of the CNS. In addition, at that time, microglial cells are activated. FACS analyses also reveal a significant increase in $CD45^{hi}CD11c^+$ dendritic cells and $CD45^{hi}D11b^+$ macrophages compared with levels in naïve and immunized AO rats. During resolution of disease in DA rats, the expression of microglia and macrophage markers is comparable with those in naïve non-immunized DA and immunized AO rats. We conclude that an initial influx of macrophages is indispensible for the development of EAE in DA rats. The presence of dendritic cells and myeloid dendritic cells at the peak of disease supports the role of these cells in EAE especially in relapses and chronicity. The activation pattern of microglia in DA rats does not indicate their role as antigen presenting cells in disease induction since they are ramified at the induction phase and only become activated after the overwhelming influx of T cells.

1. Introduction

Experimental allergic encephalomyelitis (EAE), the animal model for multiple sclerosis in humans, is a T cell mediated disease of the central nervous system characterized by local inflammatory infiltrates of myelin antigen-specific T cells and demyelination. DA and Lewis rats are highly susceptible to EAE, whereas AO, F344, PVG, and Brown Norway rats are resistant to the induction of EAE (Mostarica-Stojković et al., 1982a,b; Fournié et al., 2001) even after immunization with complete Freund's adjuvant and spinal cord homogenate (Mostarica-Stojković et al., 1982a,b). Injection of encephalitogen into the footpads of strains induces disease in the genetically susceptible Dark Agouti rats (DA) but not in Albino Oxford (AO) rats although mild inflammatory infiltrates are observed in both strains early after disease induction (Vukmanovic et al., 1990). EAE arises as a result of a Th1/Th17 response following recruitment of $CD4^+$ T helper cells which then become re-stimulated by antigen presenting cells (APCs) (Pope et al., 1998; Cua et al., 2003; Veldhoen et al., 2006; O'Connor et al., 2008; Jiang et al., 2009). It has also been demonstrated that lymphoid cells from DA and (AO × DA) F_1 hybrid rats produce significantly higher levels of interferon gamma, tumor necrosis factor and interleukin-17 than those from AO rats (Lukic et al., 1998; Miljkovic et al., 2006). Moreover, only DA rats developed EAE after the transfer of (AO × DA) F_1 hybrid encephalitogen specific cells into sub-lethally irradiated parental AO and DA hosts (Mostarica-Stojkovic et al., 1992). Using xenogeneic rodent transplant model, Jones' group (Jones et al., 2003; Robinson et al., 2008) have reported that bone marrow derived antigen presenting cells (APCs) lacking integrin expression facilitate EAE induction. The role of blood-borne and perivascular meningeal macrophages in the early phase of EAE induction has been suggested by Tran et al. (1998) who demonstrated that blocking macrophage function in the inductive phase of EAE in mice prevented the invasion of the CNS by autoreactive T cells. We therefore decided to investigate the roles of accessory cells, namely macrophages, dendritic cells, and microglia in the high susceptibility of DA rats to EAE. It has been observed that when MBP-specific T cells were passively introduced into naïve Lewis rats, T cells were detected in and around the leptomeningeal vessels where they encountered

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phagocytes which served as APC cells before the onset of clinical symptoms. These phagocytes were identified either as dendritic cells or macrophages (Bartholomäus et al., 2009). It had been previously shown that eliminating macrophages suppressed EAE in Lewis' rats (Huitinga et al., 1990, 1995; Bauer et al., 1995).

Several studies in EAE in mice have also suggested that the severity of clinical disease may reflect interaction between infiltrating T cells and the resident CNS glial cells especially microglia and astrocytes. These studies examined the apparent roles of microglial cells in EAE at its inception, with the CNS resident cells being held responsible for the antigen presentation required for the T cell clonal expansion necessary for the occurrence of disease (Issadeh et al., 1995; Gehrmann et al., 1995; Minghetti and Levi, 1998; Aloisi et al., 2000; Heppner et al., 2005; Ponomarev et al., 2007). There is however, evidence that activated microglia may downregulate Th1-induced pathology by selective ingestion of infiltrating cells leading to profound downregulation of proinflammatory cytokines involved in the autoimmune process (Magnus et al., 2001). Even more significant is the work by Greter et al. (2005) which indicated that dendritic cells (DC) and not microglia are pivotal for the development of EAE. Platten and Steinman (2005) therefore postulated that DCs acting peripherally and microglial cells acting in the perivascular compartment of the CNS, could be working in concert to ensure the development of EAE. More recently, evidence has been adduced that induction of EAE is dependent on infiltrating DCs which modulate encephalitogenic vs regulatory immune responses in the CNS (Zozulya et al., 2007). Further, it is well established that naïve encephalitogenic T cells are activated by local APCs, possibly dendritic cells to initiate epitope spreading (McMahon et al., 2005) and that CNS myeloid dendritic cells drive relapses in relapsing EAE (Bailey et al., 2007).

Using CD68, CD11c and Iba-1 immunohistochemistry and FACS analysis of $CD45CD11b^+$ and $CD11c^+$ cells, we show in this study that macrophages might be indispensible for disease development in DA rats as they appear in the CNS of immunized DA rats at the induction phase of EAE but not in EAE resistant AO rats. Finally the presence of only ramified microglia in the induction phase of the disease does not suggest their role as antigen presenting cells at the onset. Thus we provide evidence that genetically determined susceptibility to EAE may at least in part depend on early influx of macrophages into the CNS.

2. Materials and methods

2.1. Induction and evaluation of EAE

2.1.1. Animals

Seven to eight week old Dark Agouti (DA) and Albino Oxford (AO) male rats were used for all experiments. All rats were maintained in the Faculty of Medicine and Health Sciences (FMHS) animal facilities at the United Arab Emirates University. All experiments conducted were approved by the FMHS, United Arab Emirates University Animal Research Ethics Committee. Groups of rats were immunized in the left hind foot pad with 0.1 ml encephalitogenic emulsion containing rat spinal cord tissue and 100 μg of rat myelin basic protein. Animals were monitored for clinical disease starting from day 5 after inoculation. The severity of disease was assessed by grading tail, hind limb and forelimb weakness, each on a scale of 0–4 as described previously (Mostarica-Stojković et al., 1982a,b). Briefly, zero indicated no disease, 1— loss of tail tonicity, 2—hind limb weakness, 3— hind limb paralysis and 4—moribund or death. Spinal cords were excised from the spinal canal on days 10, 14 and 22 to coincide with the clinical phases of diseases namely inception, 10, most severe 14, and resolution 28 days. The level of mononuclear cell infiltration was also graded using the following semiquantitative score as described previously (Mostarica-Stojković et al., 1982a,b) as follows: 0, no infiltration; 1, mild infiltration around pial vessels; 2, single-cell infiltration within the CNS; 3, infiltration with mild perivascular cuffing; and 4, very intense infiltration with perivascular cuffing. Values obtained in groups of animals were expressed as means ± SEM.

EAE-induced AO and DA rats that were employed for histological studies were perfused with 4% paraformaldehyde solution followed by immersion in the same solution at 4 °C for 2 h. Pieces of the spinal cord were also snap frozen in liquid nitrogen for immunohistochemistry.

Pieces of paraformaldehyde-fixed spinal cords were routinely embedded in paraffin wax and 5–7.0 μm sections stained with hematoxylin and eosin (H&E) for histological studies.

2.2. Immunohistochemistry

2.2.1. Antibodies

To determine the infiltrating and CNS resident cells in the pathogenesis of the disease, we examined by immunohistochemistry the phenotype of cells at the inception, peak and resolution phases of the disease after challenge with MBP-CFA. For comparison, the spinal cords of rats that had not received any encephalitogen (naïve) were also examined. We have examined the state of activation of microglia using ionized calcium-binding protein (Iba-1) which unlike other microglia markers is expressed by all subpopulations of microglia (Shapiro et al., 2009). We also examined the distribution of astrocytes by glial fibrillary acidic protein (GFAP) (Bignami et al., 1972) immunohistochemistry as it has been reported that they may be responsible for the apoptosis of infiltrating and resident cells in the induction phase of the disease (Kohji et al., 1998). Finally, we have quantified the number of $CD4^+$ and $CD8^+$ T cells and have examined the presence of $CD68^+$ macrophages (Dijkstra et al., 1985).

Cryostat sections of thickness 75 μm cut from the DA and AO spinal cords obtained at days 10, 14 and 28 after disease induction were stained by the ABC streptavidin technique using the floating method. Frozen sections of thickness 5–6 μm were also mounted on gelatin-coated slides and stained by the ABC streptavidin method. Briefly, floating and frozen sections were incubated in 3% hydrogen peroxide in absolute methanol for 30 min to block endogenous peroxidase after which they were washed 3× for 5 min with 0.1 M PBS. The floating sections were then incubated with polyclonal rabbit ionized calcium-binding molecule (Iba-1) antibody (Wako, Germany) diluted 1:40,000 or anti-mouse glial fibrillary acidic protein (GFAP) (Sigma, St. Louis, USA) diluted 1:10,000. The frozen section slides were incubated with mouse anti-rat CD68 (Serotec, UK) for macrophages, hamster anti-rat CD11c (Pharmingen, USA) for dendritic cells and mouse anti-rat CD4 and CD8 (Serotec, UK) all diluted 1:100 in 3% Triton X in 0.1 M PBS overnight at room temperature. The following morning, the sections and slides were washed 3× for 5 min, incubated with the link antibody comprising biotinylated anti-rabbit, anti-mouse or anti-hamster IgG diluted 1:500 in 3% Triton X in 0.1 M PBS (Jackson ImmunoResearch Laboratories, Inc., USA) for 1 h and then peroxidase labeled-Extravidin (Sigma Co, St. Louis, MI, USA) diluted 1:1000 in 3% Triton X in 0.1 M PBS for 1 h, the slides being washed 3× for 5 min changes in 0.1 M PBS in between the link antibody and the Extravidin. The specimens were then washed 2× for 5 min changes of 0.1 M PBS and a third wash in 0.1 M phosphate buffer. Peroxidase activity was demonstrated with diaminobenzidine and the sections mounted on gelatin-coated slides. The sections were air-dried, dehydrated in ethanol, cleared in xylene and coverslipped using Cytoseal 60 mounting medium (Stephens Scientific, Riversdale, NJ, USA). The slides were examined on a Zeiss axiophot photomicroscope.

Floating sections from DA and AO rats were also taken 10 days after immunization and double stained by indirect immunofluorescence using fluorescein isothiocyanate (FITC) bound anti-mouse IgG and rhodamine (RRX) bound anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., USA) diluted 1:100 in 0.3% Triton X in 0.1 M PBS for 1 h. Rabbit anti-Iba-1 and mouse anti-GFAP diluted at 1:5000 and 1:1000 respectively were used as primary antibodies and sections were stained overnight at room temperature. After treatment with secondary