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Effects of pixantrone on immune-cell function in the course of acute rat experimental allergic encephalomyelitis

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Abstract

Pixantrone is an immunesuppressor similar to mitoxantrone but with lower cardiotoxicity. We evaluated the effect of pixantrone on B cells and lymphomononuclear cells in the course of acute EAE. Pixantrone reduced the number of B cells and suppressed myelin basic protein (MBP) specific IgG production. In vitro, pixantrone induced apoptosis of rat B lymphocytes in a way similar to mitoxantrone. In addition, pixantrone inhibited antigen specific and mitogen induced lymphomononuclear cell proliferation, as well as IFN-γ production, during EAE. These findings suggest a similar mechanism of action for pixantrone and mitoxantrone on the effector function of lymphomonocyte B and T cells.

Keywords: Pixantrone; Mitoxantrone; Experimental allergic encephalomyelitis; T and B cells; Myelin basic protein

1. Introduction

Experimental autoimmune encephalomyelitis (EAE), as induced by immunization with central nervous system (CNS) antigens in susceptible animals, is a T cell mediated autoimmune disease of the CNS, widely used as a model for the study of the immunopathological mechanisms of multiple sclerosis (MS) (Bert't Hart and Amor, 2003; Ridge et al., 1985; Wekerle et al., 1994). Various immunosuppressive agents have been found to be effective in preventing and treating EAE, and their effects on T and B cell responses have been elucidated (Levine and Saltzman, 1986; Mustafa et al., 1993).

Mitoxantrone is an immunosuppressive drug effective in acute EAE and recently approved for the treatment of very active MS (Goodin et al., 2003; Hartung et al., 2002). Its prolonged use is, however, limited by its cardiotoxicity (Mather et al., 1987). Mitoxantrone is a potent cytostatic drug and has various distinct immunological effects (Neuhaus et al., 2004). It inhibits the proliferation of macrophages, B and T lymphocytes both in animals (Fidler et al., 1986a,b) and in MS patients (Gbadamosi et al., 2003). It also has a complex action on the T cell function inhibiting T helper and enhancing T suppressor activities (Fidler et al., 1986b). In particular, unlike other immunesuppressants, mitoxantrone induces apoptosis of B lymphocytes in laeukemic cells (Bellosillo et al., 1998), and in peripheral blood B and T cell during MS (Chan et al., 2005). Moreover, Fidler et al. reported

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that mitoxantrone decreased secretion of pro-inflammatory cytokines (Fidler et al., 1986b). In contrast, recent ex vivo analysis of the cytokine profile of immune cells obtained from MS patients has shown a more complex pattern of action, as mitoxantrone has revealed a long-lasting decrease of IL-10 expressed in monocytes and of IL-2R-β1 expressed in T cells (Khoury et al., 2002; Gbadamosi et al., 2003). Pixantrone is an antineoplastic drug structurally related to mitoxantrone but with lower cardiac toxicity in comparison to this and other intercalating agents (Krapcho et al., 1994). The mechanism of action of pixantrone is largely unknown but is considered to be the same of mitoxantrone, as both drugs interact with DNA and with topoisomerase II. We recently demonstrated that pixantrone reduces the severity of acute EAE and decreases the relapse rate of chronic EAE in rats and mice (Cavaletti et al., 2004; Gonsette, 2004b). The drug has, therefore, an important potential role in MS treatment and its mechanism of action needs to be further investigated during EAE.

As mitoxantrone mediates a complex immune suppression involving different steps and both B and T lymphocytes, we investigated the effects of pixantrone on immune cell function in acute EAE induced in Lewis rats with the aim to provide further insight into its mechanism of action. We also compared in rat lymphomonuclear (MNC) cells the pro-apoptotic effect of pixantrone in a direct comparison with mitoxantrone.

2. Matherials and methods

2.1. EAE

Forty inbred female Lewis rats (180–200 g Harlan, Correzzana, Italy) were immunized by subcutaneous inoculation into both hind limb footpads of 50 μg of guinea pig myelin basic protein (Deibler et al., 1972) in complete Freund's adjuvant (3 mg/ml inactivated mycobacterium tuberculosis, Difco). The inoculated rats were randomly assigned to the pixantrone group (PIX, 32.5 mg/kg iv on days 3 and 7 after challenge, $n=20$) or the EAE group (sterile saline iv on days 3 and 7, $n=20$). Twenty rats were not inoculated and were used as healthy controls (CTRL). The animals were sacrificed on days 14, 23 and 41 (5 rats/group/day) post-immunization (p.i.). Blood and spleens were collected from each sacrificed animal.

2.2. Determination of serum antibodies by ELISA

Serum was obtained from 5 animals for each treatment and each day of sacrifice. Activated polystyrene ELISA plates (96 well) were coated with 1 μg/well of MBP in 0.05 M carbonate buffer (pH 9.6) and incubated at 4 °C overnight. After 5 washes with saline containing 0.05% Tween 20, plates were blocked by 10% FCS in saline Tween (100 μl/well) at RT for 60 min. Sera diluted from 1:100 to 1:100 000 were applied at 4 °C for 16 h in saline Tween 10% FCS. After 5 washes, 100 μl/well of alkaline phosphatase conjugated anti-rat IgG or IgM Fab2-specific affinity purified Ab (Sigma,Missouri,USA), diluted 1:2000 in saline Tween/FCS was added. After an overnight incubation and 5 washes, 100 μl of substrate solution consisting of 2 mg/ml *p*-nitrophenylphosphate in 10% diethanolamine buffer was applied. The reaction was blocked after 30 min with 50 μl of 1 M NaOH, and the absorbance read in a multichannel ELISA reader at 405 nm. ELISA plates, coating conditions, reagent dilutions, buffers and incubation times were tested in preliminary experiments. The results were evaluated as titers.

2.3. Cell preparation and proliferative response

Single cell suspensions were prepared from 3 to 5 spleens obtained from rats of each group in the different days and lymphomononuclear cells were isolated by ficoll hypaque gradient centrifugation. 10^5 MNC/well were cultured in 96-well plates for 3 days in the presence or absence of respectively MBP 10 μg/ml, PPD 5 μg/ml, PHA 10 μg/ml or ConA 5 μg/ml. ^{3}HThy was added in the last 8 h of culture. The data were reported as Delta-cpm (DCPM) (mean cpm stimulated cultures − mean cpm unstimulated cultures).

2.4. Spleen lymphomononuclear cell phenotypes

Spleen mononuclear cell suspensions were collected from Lewis rats (3 to 5) from CTRL, EAE and pixantrone rats at days 14, 23 and 41 p.i. Cells were washed in PBS containing 1% Fetal Calf Serum (FCS) and aliquots of 5×10^5 cells were used to measure lymphocyte subpopulations. Single aliquots were stained in the dark at 4 °C for 30 min respectively with 10 μl of: mouse anti-rat-anti-CD45RA-PE (Pan-B antibody in the rat), clone OX-33 (CALTAG, Burlingame, CA, USA) and mouse-anti-rat-anti-CD11b/c, clone OX-42 (BD Pharmingen,USA). 3×10^4 events from each sample were acquired on a four-fluorescence flow cytometer (EPICS XL cytometer, Beckman Coulter) and analyzed using Expo-32 software.

2.5. In vitro apoptosis of B cells

Single-cell suspensions were prepared from Lewis rat lymph nodes. Lymph node cells (LNC) were seeded at density of 10^5 cells/well in U-bottomed 96-well plates in RPMI 1640 medium supplemented with 5% FCS, 1 mM sodium pyruvate, 2 mM Glutamin, 50 mM β-mercaptoethanol, 15 mM HEPES, 100 U/ml penicillin and 100 mg/ml streptomycin. LNC were cultured in presence of LPS 10 mg/ml and respectively of mitoxantrone 9 nM or pixantrone 9 nM and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 42 h of culture cells were