Journal of Neuroimmunology 309 (2017) 88–99

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

B cells from patients with multiple sclerosis induce cell death *via* apoptosis in neurons *in vitro*

Robert P. Lisak[a,b,*,1], Liljana Nedelkoska[a], Joyce A. Benjamins[a,b,1], Dana Schalk[c,2], Beverly Bealmear[a], Hanane Touil[d,e,3], Rui Li[d,e,3], Gillian Muirhead[d], Amit Bar-Or[d,e,**,1,3]

[a] Department of Neurology, Wayne State University School of Medicine, Detroit, MI, USA
[b] Department of Microbiology, Immunology and Biochemistry, Wayne State University School of Medicine, Detroit, MI, USA
[c] Department of Oncology, Wayne State University School of Medicine and Karmanos Cancer Institute, Detroit, MI, USA
[d] Department of Neurology, McGill University, Montreal Neurological Institute, Montreal, Quebec, Canada
[e] Department of Neurology and Center for Neuroinflammation and Neurotherapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

ARTICLE INFO

Keywords:
B cells
Cell death
Cytokines
Multiple sclerosis
Neurons
Oligodendrocytes

ABSTRACT

B cells mediate multiple sclerosis (MS) pathogenesis by mechanisms unrelated to immunoglobulin (Ig). We reported that supernatants (Sup) from cultured B cells from blood of relapsing remitting MS (RRMS) patients, but not normal controls (NC), were cytotoxic to rat oligodendrocytes (OL). We now show that RRMS blood B cells, not stimulated *in vitro*, secrete factor/s toxic to rat and human neurons. Cytotoxicity is independent of Ig and multiple cytokines, not complement-mediated, and involves apoptosis. The factor/s have an apparent mw of > 300 kDa. B cells could contribute to damage within the central nervous system by secreting molecules toxic to OL and neurons.

1. Introduction

Studies in multiple sclerosis (MS) have continued to highlight the complexities of the pathogenesis of this disorder, particularly mechanisms underlying relentless progressive disease. MS was often described as an inflammatory demyelinating disease of the central nervous system (CNS) mainly affecting focal perivascular white matter regions, involving microglial activation, astrocyte proliferation and hyperplasia; secondary axonal changes were thought to occur as a late phenomenon. We have relearned that damage to axons occurs in the earliest stages even of relapsing remitting MS (Ferguson et al., 1997; Trapp et al., 1998). Gray matter involvement including the cerebral cortex was also viewed as involved late in disease, yet we now know that cortical gray matter damage occurs not only in chronic disease but also early in the course of the disease (Absinta et al., 2011; Bo et al., 2007; Calabrese et al., 2009; Kutzelnigg et al., 2005; Lucchinetti et al., 2011; Peterson et al., 2001; Rocca et al., 2005). The subpial pattern of cortical involvement appears to be a major component of gray matter injury highlighted in recent years as likely representing a major pathologic substrate of (non-relapsing) progressive MS, and correlating with both motor and cognitive decline better than the focal perivascular deep white matter lesions (Fisher et al., 2008; Fisniku et al., 2008; Rudick and Trapp, 2009). This subpial pathology is not perivascular and involves injury of both oligodendrocytes (OL) and neurons, evidenced in part by apoptosis of these cells in the superficial layers of cortex (Bo et al., 2006; Kutzelnigg et al., 2005; Peterson et al., 2001; Serafini et al., 2004). Of growing interest has been the potential contribution to such injury by immune cell collections in the meninges, some of which are

Abbreviations: CNS, central nervous system; DMEM, Dulbecco's modified essential medium; DMT, disease modifying therapy; EBV, Epstein-Barr virus; FGF, fibroblast growth factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; Ig, immunoglobulin; IL, interleukin; kDa, kilodalton; LT, lymphotoxin; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; MS, multiple sclerosis; mw, molecular weight; NC, normal control; OL, oligodendrocyte; OPC, oligodendrocyte precursor cell; PBS, phosphate buffered saline; PPMS, primary progressive multiple sclerosis; RRMS, relapsing remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; Sup, supernatant; TGF, transforming growth factor; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl dUTP nick end labeling

* Correspondence to: R.P. Lisak, Department of Neurology, Wayne State University School of Medicine, 8D, University Health Center 4201 St Antoine, Detroit, MI 48201, USA.
** Correspondence to: A. Bar-Or, Perelman Center for Advanced Medicine (PCAM) South Pavilion, 7th Floor; Rm 7633400 Civic Center Blvd, Philadelphia, PA 19104, USA
E-mail addresses: rlisak@med.wayne.edu (R.P. Lisak), lnedelk@med.wayne.edu (L. Nedelkoska), jbenjami@med.wayne.edu (J.A. Benjamins), DLS2DA@hscmail.mcc.virginia.edu (D. Schalk), bbealmea@med.wayne.edu (B. Bealmear), hanane.touil@mail.med.upenn.edu (H. Touil), rui.li@mail.med.upenn.edu (R. Li), g.muirhead@gmail.com (G. Muirhead), amitbar@upenn.edu (A. Bar-Or).
[1] Drs. Lisak, Benjamins and Bar-Or contributed equally to this study.
[2] Current address: Department of Medicine, Division of Hematology/Oncology, University of Virginia, Charlottesville, VA, USA.
[3] Current address: Center for Neuroinflammation and Neurotherapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

http://dx.doi.org/10.1016/j.jneuroim.2017.05.004
Received 7 February 2017; Received in revised form 9 May 2017; Accepted 10 May 2017

described as 'B cell rich' (Choi et al., 2012; Kutzelnigg et al., 2005; Magliozzi et al., 2007; Serafini et al., 2004).

We have been interested in the effector role of B cells in causing damage to OL/myelin and neurons/axons independent of involvement of immunoglobulin (Ig) and/or complement. We previously reported that secretory products from cultures of B cells (including unstimulated B cells) from blood of untreated patients with RRMS are cytotoxic for OL *in vitro* in mixed rat glial cultures, whereas Sup from B cells from normal controls (NC) are not cytotoxic (Lisak et al., 2012). We hypothesize that B cells entering the meninges and cerebral spinal fluid (CSF) from the peripheral immune system could secrete factors that lead to damage characteristic of MS (Fischer et al., 2013; Haider et al., 2016; Lassmann, 2012; Lassmann, 2014) in the underlying cortical gray matter.

In the current study we tested whether Sup from blood-derived B cells of patients with RRMS seen at the Montreal Neurological Institute/McGill University also compromise neuronal viability in comparison to Sup of B cells from NC and examined the mechanism underlying such toxicity if observed. We hypothesized that neuronal cell death would be induced by the MS patient-derived B cell Sup and this death would be due to apoptosis. We examined the effects of B cell Sup from RRMS patients and NC on both rodent and human neurons *in vitro*. We measured levels of IgM and IgG and an expanded number of cytokines and related protein secretory products of inflammatory cells in the B cell culture Sup.

2. Methods

2.1. Materials

Human neurons (#1520, medium (#1521), growth supplement (#1562), and penicillin streptomycin solution (#0503) were purchased from Sciencell (San Diego CA). Poly-D-lysine (P7405) and laminin (L2020) were from Sigma (St. Louis MO).

ELISA kits were obtained as follows: 14-PLEX magnetic bead custom array (HCYTOMAG60K-14) from EMD Millipore; 25-PLEX non-magnetic cytokine array (LHC00009) from Life Technologies/Invitrogen; transforming growth factor-β (TGF-β), LEGEND MAX (436707) and human interleukin (IL)-35 heterodimer, LEGEND MAX (439507) from BioLegend; lymphotoxin-α (LT-α) (MBS268485) and lymphotoxin-β (LT-β) (MBS261430) from MyBiosource; IL-16 and IL-23 2-PLEX (HCYP2MAG-62K-02) from EMD Millipore; and matrix metalloproteinase-9 (MMP-9) (DMP900) from R & D. ELISA assays for Fas (apo-1; ab100513); Fas ligand (ab100515); CD40 (ab119516); CD40 ligand (ab196268) and granzyme B (ab46142) were from Abcam. The complete list of cytokines and biological modifier proteins examined are listed in Table 1.

2.2. Rat neuronal cultures

Neurons were cultured from neonatal rat brain based on the method of Eide and McMurray (Eide and McMurray, 2005), modified as described (Lisak et al., 2011). For each explant, three forebrains were used; each half forebrain was minced in 2 ml of HEPES buffered saline containing 2 mg/ml of papain, then combined with the other tissue for the subsequent steps. The final cell pellet was triturated 5–7 times in 3 ml of plating medium; 50 μl were plated on each coverslip, with 10 coverslips per 60 mm dish. After overnight attachment at 37^{0}C, 2 ml of plating medium with 100 ng/ml nerve growth factor was added to each dish. Cytosine arabinoside, final concentration 1×10^{-5} M, was added the next day; after 5 days, the medium was changed to B-27 feeding medium containing NGF and cytosine arabinoside. Neuronal cultures were used to test toxicity of B cell Sup between 5 and 7 days after explant. Cultures were 85–90% neurons, with the remaining cells astrocytes and microglia, and no detectable OL based on phenotypic markers (Lisak et al., 2011; Lisak et al., 2012).

Table 1
Cytokines and factors assayed in B cell supernatants from NC and RRMS.

Cytokine (14-PLEX)	Cytokine (25-PLEX)		Individual ELISAs
GM-CSF	Eotaxin	IL-10	CD40
IFNα	FGF (basic)	ILp40p70	CD40 ligand
IL-1β	GM-CSF	IL-13	Fas
IL-4	IFN-α	IL-15	Fas ligand
IL-6	IFN-γ	IL-17	Granzyme B
IL-10	IL-1β	IP-10	IL-16
IL-12p40	IL-1RA	MCP-1	IL-23
IL-12p60	IL-2	MIG (CXCL9)	LT-α
MIP-1α (CCL3)	IL-2R	MIP-1α (CCL3)	LT-β
MIP-1β	IL-4	MIP-1β	MMP-9
TGF-α	IL-5	RANTES (CCL5)	TGF-β
TNF-α	IL-6	TNF-α	
TNF-β	IL-7	VEGF	
VEGF	IL-8		

Abbreviations: FGF, fibroblast growth; GM-CSF, granulocyte-colony stimulating factor; IFN, interferon; IL, interleukin; IL-1RA, interleukin 1 receptor; IL-2R, interleukin-2 receptor; LT, lymphotoxin; MCP, monocyte chemoattractant protein; MIG, monocyte-induced by gamma interferon; MIP, macrophage inflammatory protein; MMP-9, matrix metalloproteinase; TGFβ, transforming growth factor-alpha; TNF, tumor necrosis factor; RANTES, regulated on activation, normal T cell expressed and secreted; VEGF, vascular endothelial growth factor.

2.3. Human neuronal cultures

Coverslips previously coated with poly-D-lysine (1 mg/10 ml) were placed in a 24 well plate and coated overnight with laminin (10 μg/ml); the excess solution was removed just prior to plating the cells. One vial of cryopreserved primary human neurons (Sciencell) containing 1×10^6 cells was thawed and suspended in 14 ml of complete neuronal medium, per the supplier's instructions (Sciencell), then plated in 700 μl aliquots. After 4 days in culture, fresh medium was added, and the neurons were used the next day to test the B cell Sup.

2.4. Rat mixed glial cell cultures

Some of the B cell supernatants from RRMS patients and NC used in this study were tested previously for capacity to kill OL in rat mixed glial cell cultures (Lisak et al., 2012). We tested additional newly produced supernatants from RRMS patients ($n = 6$) and NC ($n = 6$) to ascertain whether these supernatants were also toxic to OL. Cultures enriched in differentiated OL were prepared from neonatal rat brain, using a modification of the shakeoff method (McCarthy and de Vellis, 1980); cultures from the 3rd–5th shakeoffs were used, resulting in cultures containing on average 35–40% OL, 35–40% astroglia and 15% microglia. The methods for preparation of cultures have been previously described in detail (Lisak et al., 2006; Lisak et al., 2012).

2.5. B cell cultures

Blood was obtained with informed consent from patients with RRMS and age matched controls at the Montreal Neurological Institute/McGill University. B cells were obtained by positive selection for CD19 from peripheral blood of consenting patients with RRMS (Polman et al., 2011) and from NC, as previously described (Bar-Or et al., 2010; Bar-Or et al., 2001; Duddy et al., 2007; Duddy et al., 2004) and as approved by the Ethics Review Board of the Montreal Neurological Institute and McGill University. B cells were isolated by density centrifugation using Ficoll (GE Healthcare). CD19 beads (Miltenyi Biotech) were used to positively select B cells according to the manufacturer's protocol. Purity of the cells was assessed using flow cytometry with an enrichment of > 98%. B cells were then cultured without any stimulation for 3 days (Bar-Or et al., 2010; Bar-Or et al., 2001; Duddy et al., 2004; Lisak et al., 2012); Sup were harvested and frozen at -80° C until testing for cytotoxicity studies performed at Wayne State University School of