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AVALIAÇÃO DA IMUNOMODULAÇÃO DE CITOCINAS E COMPONENTES APOPTÓTICOS POR GANGLIOSÍDEOS NA ENCEFALOMIELITE EXPERIMENTAL AUTOIMUNE.

Tese apresentada ao curso de pós-graduação da Faculdade de Ciências Médicas para obtenção do título de Doutor em Clínica Médica, na área de Ciências Básicas

Orientador: Prof. Dr. Ricardo de Lima Zollner

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SUMÁRIO

Lista de abreviaturas

AICD	*Activation-induced cell death*
AIF	Fator indutor de apoptose/ *apoptosis inducing factor*
APC	Células apresentadoras de antígeno/*Antigen-presenting cell*
AU	*Arbitrary units*
BHE	Barreira hematoencefálica
CEMIB	Centro Multidisciplinar de Investigação Biológica
CFA	Adjuvante completo de Freund/*Complete Freund's Adjuvant*
CNS	*Central nervous system*
DPI	Dias pós-inoculação/ *Days post-immunization*
EAE	*Encefalomielite experimental autoimune/Experimental autoimmune encephalomyelitis*
EM	Esclerose múltipla
GP-MBP	Proteína básica de mielina de cobaia/*Guinea pig myelin basic protein*
IFN	Interferon
IL	Interleucina/*Interleukin*
MBP	Proteína básica de mielina/*myelin basic protein*
MHC	Complexo de histocompatibilidade principal/*major histocompatibility complex*
MMP	Metaloproteinase de matriz/*Matrix metalloproteinase*
MOG	Glicopreoteína de mielina do oligodendrócito/*Myelin-oligodendrocyte glicoprotein*
mRNA	RNA mensageiro/*Messeger RNA*
MS	*Multiple sclerosis*
Mt	*Mycobacterium tuberculosis*
NF-κB	Fator nuclear-κB/*Nuclear factor-κB*
NGF	*Fator de crescimetno nervoso/ Nerve growth factor*
NK	*Natural killer*
NO	*Óxido nítrico/Nitric oxide*
PKC	*Proteinaquinase C*
PLP	Proteína proteolipídica/*Proteolipid protein*
RNA	Ácido ribonucleico/*ribonucleic acid*
RT-PCR	Reação de polimerase em cadeia por transcriptase reversa/ *Reverse transcriptase-polymerase chain reaction*
SE	*Semiquantitative expression*
SNC	Sistema nervoso central
TGF	*Transforming growth factor*
Th1	T auxiliar/T helper1
Th2	T auxiliar/T helper2
TNF	Fator de necrose tumoral/*Tumor necrosis factor*
TNF-R1	*Receptor 1 TNF*
TUNEL	*Terminal deoxybucleotidyl transferase mediated dUTP nick-end labeling assay*
V-CAM	Molécula de adesão celular vascular/*Vascular – Cell adhesion molecule*
VLA-4	*Very late antigen-4*

Resumo

A esclerose múltipla é doença inflamatória crônica do sistema nervoso central (SNC), na qual linfócitos T auto-reativos voltam-se contra os antígenos da mielina. A doença caracteriza-se por surtos e remissões, que levam a deficiências neurológicas irrecuperáveis. A resposta inflamatória aberrante sugere etiologia autoimune para esclerose múltipla, durante a qual, acredita-se que linfócitos T $CD4^+$ ativados contra antígenos da mielina atravessem a barreira hematoencefálica (BHE), iniciando reação imunológica, seguida de indução e liberação de citocinas, produção de anticorpos e ativação de células da micróglia e astrócitos. Por apresentar inúmeras similaridades com a esclerose múltipla, a encefalomielite experimental autoimune (EAE), tem sido usada como modelo experimental para o estudo desta doença. A EAE pode ser facilmente induzida utilizando-se proteínas da mielina na *Indução Ativa*, ou infundindo-se linfócitos T reativos contra estas proteínas na *Transferência Adotiva*. Assim como observado na esclerose múltipla, linfócitos T $CD4^+$ são as células efetoras na EAE, liberando citocinas e recrutando outras células do sistema imunológico para o ataque ao sistema nervoso central. Neste processo, citocinas têm papel preponderante na modulação da EAE, fornecendo os sinais necessários para ativar linfócitos T específicos para auto-antígenos. Dada sua natureza neurotrófica e imunomodulatória, tem sido proposto o uso de gangliosídeos na redução das conseqüências deletérias de várias doenças neurodegenerativas e de origem autoimune. Os gangliosídeos possuem atividades imunomodulatórias múltiplas diminuindo respostas linfoproliferativas e modulando a produção de citocinas. No presente trabalho, utilizando-se ratos da linhagem Lewis no modelo da EAE aguda,

foram avaliados os efeitos da administração de gangliosídeos na alteração do perfil de expressão de citocinas Th1 para Th2/Th3 e na indução de apoptose em células esplênicas e medula espinhal respectivamente, durante os sinais clínicos e após a recuperação da doença. Os resultados demonstram que o grupo tratado com gangliosídeos exibe doença moderada, com a expressão gênica de IFN-γ diminuída e de TGF-β elevada, sugerindo que os gangliosídeos estudados possam modular a síntese de citocinas Th1, alterando o perfil para o fenótipoTh2/Th3. Por sua vez, esta alteração do perfil de citocinas pode regular moléculas relacionadas à apoptose, fornecendo evidências que os gangliosídeos diminuam a expressão *in vivo* de Bcl-2 e Bcl-W, juntamente com o aumento da expressão gênica de Fas e FasL.

Abstract

The multiple sclerosis is a chronic inflammatory disease of the central nervous system (SNC), which autoreactive T cells drive immune response against myelin antigens. The relapses and remissions of the disease are responsible for the permanent neurological deficits. The observation of an aberrant inflammatory response against myelin epitopes suggests autoimmune etiology for multiple sclerosis. During the inflammatory process, CD4 T cells activated against myelin antigens cross the blood-brain barrier (BBB) beginning an autoimmune reaction, followed by the release of cytokines, antibodies production and activation of the microglia and astrocytes. By present similarities with the multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE), has been used as experimental model for the study of multiple sclerosis pathology. EAE can be actively induced with myelin proteins, or by infusion of T cells driven against these proteins in the adoptive transfer. As observed in the multiple sclerosis, $CD4^+$ T lymphocytes are the effector cells that release cytokines and recruit other immune cells for the attack to the central nervous system. In this process, cytokines has a crucial role in the EAE modulation, supplying the signs to activate autoreactive T cells. By their neurotrophic and immunomodulatory properties gangliosides have been proposed to reduce deleterious consequences of many neurodegenerative and autoimmune diseases. In the present work, the effects of gangliosides were tested on the switching of Th1 to Th2/Th3 cytokine expression and apoptosis induction, in spleen cells and spinal cord respectively, from Lewis rats on the acute EAE, during clinical signs and after recovery from the disease. The results demonstrate that the group treated with

gangliosides show mild disease, with low expression of IFN-gamma mRNA and high TGF-beta mRNA expression, suggesting that the gangliosides may modulate Th1 cells by the shifting the profile to the Th2/Th3 phenotype. Moreover, the results demonstrate that ganglioside treatment leads to an increase in TGF-beta levels in brain and spleen cells, together with an increase in Fas and FasL mRNA expression.These results provide the evidence that the ganglioside action on the Bcl-2, Bcl-W, Fas, and FasL expression is associated with cytokine modulation.

INTRODUÇÃO

1.1 Esclerose Múltipla

A esclerose múltipla, é a doença inflamatória crônica mais comum do sistema nervoso central (SNC), afetando aproximadamente 1 milhão de pessoas em todo mundo, com predomínio em mulheres na proporção de 2:1(DYMENT and EBERS, 2002).

Os primeiros sintomas surgem no início da fase adulta sob a forma surto-remitente (STEINMAN, 2001), caracterizando-se por episódios de disfunção neurológica, seguida por períodos de estabilização ou remissão. Os sintomas podem desaparecer ou tornarem-se permanentes (STEINMAN, et al. 2002).

Com o tempo, a maioria dos pacientes com o quadro surto-remitente desenvolve lenta e progressiva deterioração neurológica, independente dos surtos clínicos, denominada de Esclerose Múltipla Secundária Progressiva. Entretanto, aproximadamente 10% dos pacientes, apresentam doença caracterizada pelo curso progressivo desde o início, com eventuais surtos, denominada Esclerose Múltipla Primária Progressiva (KEEGAN and NOSEWORTHY, 2002; HEMMER et al. 2002; PENDER and WOLFE, 2002).

Os sintomas mais comuns são, perda de visão causada pela neurite óptica, paresia e paralisia, ataxia, fadiga e incontinência. Alguns pacientes podem, ainda, apresentar perda cognitiva e dificuldades de concentração e memória (EWING and BERNARD, 1998).

Diversos fatores indicam que a interação entre fatores genéticos e ambientais contribua para o desenvolvimento da esclerose múltipla. Estudos demonstram que a susceptibilidade à doença tem caráter poligênico, envolvendo o complexo de

histocompatibilidade principal (MHC) e diversos *loci* não-MHC.(AMOR, et al.1997).

Com relação aos fatores ambientais, estudos epidemiológicos têm demonstrado que os surtos da EM estão frequentemente associados a infecções virais. Além disso, há forte correlação entre latitude e incidência da esclerose múltipla, documentada tanto no hemisfério norte, onde se observa maior incidência em latitudes setentrionais, como no hemisfério Sul, conforme estudos realizados na Austrália e Nova Zelândia, demonstrando que a incidência é sete vezes maior na Tasmânia (região sub-tropical), que em Queensland (região tropical). Dados obtidos a partir de estudos de migração populacional, sugerem que o risco é conferido pela exposição ao meio ambiente durante adolescência e que a migração antes da puberdade de uma área de prevalência elevada para uma de prevalência baixa, resulta na redução do risco de desenvolvimento da esclerose múltipla (SADOVNICK and EBERS, 1993; SCOLDING, et al. 1994;.LYNCH and ROSE, 1996; EWING and BERNARD, 1998).

1.1.1 Histopatologia

A EM apresenta lesões agudas com bainhas perivasculares formadas por linfócitos T e B. A presença persistente de anticorpos reflete a ativação de plasmócitos no SNC, resultando no aparecimento de bandas oligoclonais de imunoglobulina no líquor. A destruição dos oligodendrócitos é dependente do tipo de lesão (ativa precoce, ativa tardia e crônica).

Observa-se que a destruição de oligodendrócitos nas lesões ativas precoces é pequena, entretanto, nos estágios tardios, mais de 80% da população destas células é perdida (SCOLDING, et al. 1994; AMOR, et al., 1997).

Embora as lesões da EM afetem predominantemente a mielina do SNC, estudos têm demonstrado a transecção axonal nas lesões ativas. A perda neuronal ocasionada pode ser o principal fator incapacitante a longo prazo, implicando em seqüelas definitivas, (KEEGAN and NOSEWORTHY, 2002).

Várias características imunológicas e patológicas sugerem etiologia autoimune, na qual proteínas da mielina atuam como antígenos (BRIGHT and SRIRAM, 2001; GALBOIZ and MILLER, 2002). Assim, a esclerose múltipla é tida como exemplo de resposta inflamatória aberrante na qual a bainha de mielina torna-se o alvo do ataque imunológico (RAINE, 1994.).

Acredita-se que linfócitos T $CD4^+$ do tipo auxiliar 1 (Th1) ativados contra antígenos da mielina, atravessem a barreira hematoencefálica (BHE) iniciando reação imunológica, seguida pela indução e liberação de citocinas, produção de anticorpos e ativação de células da micróglia e astrócitos (BASHIR and WHITAKER, 1998).

Embora o evento primordial que leva a manifestação da EM seja ainda desconhecido, é quase certo que a ativação inicial das células imunes ocorra nos tecidos linfóides. Proteínas antigênicas que apresentam reação cruzada com proteínas do SNC (hipótese autoimune), um patógeno residente no cérebro (hipótese da infecção) ou proteínas do SNC após degeneração primaria (hipótese da degeneração) são liberadas na periferia. Estas proteínas alcançariam linfonodos e baço desencadeando resposta imunológica específica (HEMMER et al., 2002).

Muitas proteínas microbianas exibem homologia com estruturas encontradas na bainha de mielina, levando ao ataque autoimune contra os antígenos da mielina,

por mimetismo molecular (WUCHERPFENNIG and STROMINGER, 1995).

Uma vez ativada na periferia, a resposta inflamatória induz a liberação de interferon-γ (IFN-γ) e fator de necrose tumoral-α (TNF-α) que, por sua vez, promovem a expressão da molécula de adesão celular vascular (V-CAM) e das moléculas do complexo de histocompatibilidade principal classe II (MHC-II) nas células endoteliais de capilares do SNC. Linfócitos ativados podem atravessar o endotélio cerebral através da expressão de moléculas de adesão, como o VLA-4 (very late antigen-4), ligando-se a outras moléculas de adesão presentes no endotélio inflamado como a V-CAM, iniciando a diapedese e quebrando, dessa forma, o primeiro elemento da BHE. Uma vez que os linfócitos tenham atravessado o endotélio e entrado em contato com o colágeno tipo IV da membrana basal do endotélio vascular, as células do sistema imunológico secretam enzimas como metaloproteinases de matriz (MMP) destruindo a membrana basal e permitindo o acesso de linfócitos T ativados à substância branca do SNC (STEINMAN et al., 2002).

1.2 Encefalomielite Experimental Autoimune - EAE

Por apresentar diversas similaridades, a encefalomielite experimental autoimune (EAE), é amplamente utilizada como paradigma experimental para o estudo da esclerose múltipla.

Dos modelos utilizados para indução da EAE, aproximadamente 58% têm sido realizados em ratos e cerca de 42% em camundongos, indicando a aceitação ampla da doença induzida em ratos. O modelo de indução ativa tem sido utilizado para estudos da imunopatogênese e de imunoterapia da EAE (LINK and XIAO, 2001).

Como observado na esclerose múltipla, fêmeas apresentam maior susceptibilidade para EAE, na proporção de 2:1. Esta susceptibilidade está relacionada com os *loci* do complexo de histocompatibilidade principal (MHC) (STEINMAN, 1999)

A EAE pode ser facilmente induzida em diferentes espécies de animais susceptíveis pela imunização com homogeneizado total de tecido nervoso, ou com componentes purificados do sistema nervoso central, como por exemplo, a proteína básica de mielina (MBP), proteína proteolipídica (PLP), glicoproteína de mielina do oligodendrócito (MOG), assim como peptídeos encefalitogênicos destas proteínas. Para aumento da resposta imunológica o inóculo é preparado com Adjuvante Completo de Freund (CFA), suplementado com *Mycobacterium tuberculosis* morta. O óleo do adjuvante envolve a proteína aumentando sua apresentação local. Este tipo de indução é denominado *Indução Ativa* (GOLD et al., 2000; PETRY et al., 2000).

Estas proteínas são, também, utilizadas para produção de linfócitos T auxiliares do tipo 1 (Th1), no modelo de indução passiva da EAE, através de transferência adotiva de células encefalitogênicas. A doença se desenvolve de forma mais rápida, mas semelhante à imunização ativa (GOVERMAN and BRABB, 1996).

Os sinais clínicos da EAE manifestam-se de forma ascendente, iniciando com a perda de tônus da cauda progredindo para paralisia dos membros traseiros, paralisia de membros traseiros e dianteiros e, finalmente, morte. No rato Lewis a doença em geral ocorre na forma monofásica, apresentando os primeiros sinais dentro de 10 a 12 dias após a imunização, recuperando-se 18 a 20 dias após a imunização, estes animais tornam-se resistentes à nova indução da doença

(WILLENBORG, 1979).

A histopatologia da EAE compreende inflamação do SNC com infiltrados linfocitários peri-vasculares distribuídos por vários focos na substância branca, com graus variados de desmielinização. Linfócitos T CD4+ são as células efetoras que reconhecem os antígenos da mielina. As lesões ocorrem predominantemente na medula espinhal, embora possam ocorrer no cérebro (GOVERMAN and BRABB, 1996; CONSTANTINESCU, et al., 1998).

Linfócitos T $CD4^+$ ativados atravessam a BHE penetrando no SNC, onde a micróglia, células apresentadoras de antígeno (APCs) residentes, apresentam epítopos endógenos da mielina. Desta forma, linfócitos T CD4+, micróglia e astrócitos ativados liberam quimiocinas e citocinas que induzem um grande influxo de monócitos periféricos para o parênquima do SNC. A lesão é decorrente da fagocitose da bainha de mielina por macrófagos/micróglia e pelos efeitos citotóxicos de moléculas efetoras, como citocinas Th1 (TNF-α, IFN-γ) e óxido nítrico (MILLER and SHEVACH, 1998).

1.3 Citocinas

Citocinas têm papel crucial no início, progressão e regulação da lesão autoimune. Vários tipos de reações imunológicas podem ser definidas pelo perfil destas proteínas. Da mesma forma, a adição ou inibição de citocinas específicas pode alterar de maneira significativa a resposta imunológica (MOSMANN et al., 1989).

Além de atuarem na modulação da autoimunidade, citocinas e seus receptores são ubíquos no SNC, demonstrando que estas moléculas agem nas

funções fisiológicas tais como o sono e a plasticidade sináptica. A maior parte da expressão de citocinas observada no SNC tem origem em astrócitos e micróglia (JAFARIAN-TEHRANI and STERBERG, 1999).

Após o reconhecimento dos antígenos encefalitogênicos, linfócitos T diferenciam-se no fenótipo Th1, com perfil de citocinas pró-inflamatórias como TNF-α, IFN-γ e IL-12. Há evidências que sugerem o envolvimento destas citocinas na promoção da EAE e esclerose múltipla. Por outro lado, a fase de recuperação/remissão está relacionada com a expressão do fenótipo Th2/Th3, com liberação das citocinas IL-4, IL-10 e TGF-β (HILL and SARVETNICK, 2002)

O estudo da EAE aguda monofásica em ratos Lewis demonstrou que o aparecimento dessas citocinas é seqüencial. Inicialmente, antes dos sinais clínicos, ocorre a produção de IL-12 pelas células apresentadoras de antígeno coincidindo com o surgimento das primeiras células inflamatórias. Esta citocina promove a diferenciação das células Th1, que secretam IFN-γ , TNF-α e/ou LT-α.

Posteriormente, na fase aguda da doença e paralelamente ao aumento da gravidade dos sinais clínicos, também são liberadas IFN-γ e TNF-α, desencadeando a doença por lesão direta aos oligodendrócitos e à mielina, bem como pela ativação de macrófagos, que estão envolvidos na desmielinização.

Durante a fase de recuperação da EAE ocorre o aumento da expressão de citocinas provenientes do fenótipo Th2 (IL-4, IL-10 e IL-13) que atuam como inibidores de macrófagos, juntamente com a expressão de TGF-β, produzido por células Th2/Th3. Contudo, a expressão de IL-4 não tem relação clara com a seqüência dos eventos patológicos (OLSSON, 1995; NAVIKAS & LINK, 1996;

BEGOLKA & MILLER, 1998).

1.4 Apoptose

Outro fator importante na regulação da EAE é a ocorrência de apoptose induzida por Fas/FasL. O receptor Fas (CD95/APO-I) é uma glicoproteína de superfície membro da família de receptores do TNF/fator de crescimento nervoso (NGF). Pode ser facilmente induzido por citocinas tais com o TNF-α e IFN-γ, quando não constitutivamente expresso. Em sua porção citoplasmática o Fas exibe uma região denominada "domínio da morte", com homologia no TNF-R1, este domínio é suficiente para transdução do sinal apoptótico (NAGATA and GOLDSTEIN, 1995).

O Fas ligante (FasL) tem distribuição restrita, sendo constitutivamente expresso principalmente em órgãos ricos em linfócitos T. Sua expressão pode ser aumentada em linfócitos T citotóxicos, células "natural Killer" (NK) e linfócitos Th1. Linfócitos T ativados podem gerar as duas formas de FasL, a solúvel e a ligada à membrana plasmática (TANAKA et al., 1995).

Na EAE, a participação de Fas/FasL tem sido demonstrado por observações feitas em camundongos B6.*lpr* (mutante para expressão de Fas) e camundongo B6.*gld* (mutante para expressão de FasL) os quais apresentam certa resistência ao desenvolvimento dos sinais clínicos da EAE (WALDNER et al., 1997; SABELKO et al., 1997). O mecanismo proposto para este envolvimento, refere-se a expressão de FasL em células ativadas no sistema nervoso central e sua interação com Fas expresso em oligodendrócitos, provavelmente estimulado por IFN-γ, levando à morte celular. Como conseqüência, linfócitos T auto-reativos adicionais, macrófagos e outros tipos celulares seriam recrutados para o sistema nervoso central

estabelecendo, desta forma, a lesão inflamatória/imune (DITTEL, 2000).

Estes mesmos estudos indicam que Fas e FasL podem modular a resposta autoimune limitando a expansão de populações de linfócitos auto-reativos através da apoptose (SABELKO-DOWNES, *et al.*, 1999).

Bonetti e colaboradores (1997), avaliando a ocorrência de apoptose por Fas/FasL em camundongos SJL/J, demonstraram que as células mortas por este sistema são células inflamatórias e da micróglia, tanto em lesões agudas, como em lesões crônicas. Estes achados sugerem que Fas/FasL atuam como reguladores da resposta imunológica, eliminando células efetoras recrutadas e residentes do SNC.

Na EAE aguda, linfócitos T sofrem morte celular programada no SNC. O número máximo de células apoptóticas ocorre durante a fase de recuperação, sugerindo que a eliminação de linfócitos T encefalitogênicos seja um dos mecanismos envolvidos na recuperação espontânea e no desenvolvimento da tolerância (PENDER, 1999).

1.5 Gangliosídeos

A expansão do conhecimento dos mecanismos envolvidos na EAE/Esclerose Múltipla tem permitido o desenvolvimento de diversos agentes terapêuticos. Alguns possuem propriedades imunossupressoras não específicos e outros mais seletivos, contudo, todos procurando limitar a resposta imunológica (BASHIR and WHITAKER, 1998).

O aspecto inflamatório das lesões na esclerose múltipla tem sido o principal foco das estratégias terapêuticas, dada a facilidade de indução da EAE, que

reproduz a lesão neurológica imuno-mediada. Na elaboração de estratégias terapêuticas, novas abordagens devem ser adotadas, para inibir a desmielinização, evitar a perda neuronal, proteger os axônios ou promover a remielinização (LUCCHINETTI et al., 2001).

Neste contexto, os gangliosídeos a partir de suas propriedades têm sido utilizados na terapia no tratamento de lesões do sistema nervoso central, baseado em suas características neurotróficas e neurotrópicas. Além disso, a literatura tem demonstrado que esses compostos atuam, de forma imunomodulatória, suprimindo a reposta imunológica.

Gangliosídeos constituem família de moléculas anfipáticas que variam entre 1300 a 2500 unidades de massa atômica (AMU), com uma cauda hidrofóbica de ceramida inserida no folheto externo da membrana plasmática e uma cabeça hidrofílica glicídica, com um ou mais resíduos de ácido siálico que se protraem no espaço extracelular. (MÖBIUS et al., 1999;RAVINDRANATH et al., 2001)

Gangliosídeos compõem a maioria das membranas celulares de seres eucarióticos, onde estes glicoesfingolípides possuem importante função estrutural (ECHTEN e SANDHOFF; 1993), atuando como receptor e co-receptor para muitos agentes bioativos como citocinas, hormônios, toxinas e vírus. Estão envolvidos ainda na diferenciação, reconhecimento e interação celular e na regulação do crescimento (BERGELSON, 1995).

Estes complexos glicoesfingolípides possuem ainda, ação moduladora sobre o sistema imune. Diversas evidências demonstram que estes compostos podem inibir a ativação e diferenciação de linfócitos T, a expressão da molécula de CD4 e

proliferação de linfócitos B, T, monócitos, macrófagos e células NK (OFFNER et al., 1987; CHU and SHAROM, 1995; DOZMOROV et al., 1997). Presentes em grandes quantidades nas células tumorais e no sistema nervoso central, os gangliosídeos estão associados à imunossupressão observada nestes tecidos (MASSA, 1993; RAVINDRANATH et al., 2001).

Estes compostos podem bloquear de forma seletiva a produção de citocinas pró-inflamatórias como IFN-γ, TNF-α e IL-2 (TH1) e, desta forma, inibir a proliferação de células T impedindo a entrada desta célula no ciclo celular. Por outro lado, as citocinas antiinflamatórias como IL-4, IL-10 e TGF-β podem ter sua síntese estimulada por estes compostos (IRANI et al., 1996).

A função de gangliosídeos na indução da apoptose não está totalmente esclarecida. Contudo, o acúmulo de gangliosídeos está associado a apoptose mediada por Fas e ceramidas, pela associação gangliosídeo-mitocôndria, levando à ativação de caspases através da liberação de fatores apoptogênicos (DE MARIA et al., 1997; GARCÍA-RUIZ et al., 2000).

Baseado nos dados expostos acima , o presente trabalho teve por objetivo avaliar a ação imunomodulatória de gangliosídeos sobre a evolução clínica da EAE, bem como avaliar a expressão de citocinas pró e antiinflamatórias e moléculas relacionadas com apoptose.

TH1 AND TH2 CYTOKINE IMMUNOMODULATION BY GANGLIOSIDES IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Objetivos:

1. Investigar a ação do tratamento com gangliosídeos sobre a manifestação das deficiências neurológicas da EAE.
2. Avaliar a ação de gangliosídeos sobre a viabilidade de células esplênicas mantidas em cultura com estímulo de GP-MBP e gangliosídeos.
3. Analisar o efeito modulador de gangliosídeos sobre a expressão do mRNA de citocinas pró e antiinflamatórias, em células esplênicas nas diferentes fases da EAE (fase de sinais clínicos e fase de recuperação).

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Th1 and Th2 cytokine immunomodulation by gangliosides in experimental autoimmune encephalomyelitis

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In press

Abstract

In experimental autoimmune encephalomyelitis, a classical model for multiple sclerosis, the cytokines provide the necessary signals to activate specific T cells for self-antigens. Gangliosides have multiple immunomodulatory activities, decreasing the lymphoproliferative responses and modulating cytokine production. Here, we tested the effects of gangliosides on the switching of Th1 to Th2 cytokine expression, in spleen cells obtained from Lewis rats during the acute phase of EAE, and after recovery from the disease. For this purpose, total RNA from spleen cells was isolated and submitted to RT-PCR to investigate Th1 (IL-2, TNF-alpha, and IFN-gamma) and Th2/Th3 (IL-10 and TGF-beta) cytokine gene expression. Results demonstrate that the group treated with gangliosides displays mild disease, with low expression of IFN-gamma mRNA and high TGF-beta mRNA expression. We conclude that the gangliosides may modulate Th1 cells by the synthesis of cytokines shifting the profile to the Th2/Th3 phenotype.

Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). CD4+ Th1 cells initiate the MS inflammatory process, which leads to a secondary macrophage recruitment, and subsequent myelin destruction [1,2]. These cells cross the blood-brain barrier starting an immune reaction, which subsequently attracts a variety of non-specific cells. Cytokine induction and release, and antibody production are followed by the activation of microglia and astrocytes [3,4]. Together, these events constitute a chronic inflammatory and demyelinating disease, resulting in CNS injury with varying degrees of neurological deficit [5]. The subject of cytokines has become a central topic within MS research. Evidence exists to suggest that these molecules play an essential role in the pathophysiology of MS, both by regulating the aberrant autoimmune response and by mediating myelin damage [6].

This disease mechanism is similar to that found in experimental autoimmune encephalomyelitis (EAE), an experimental paradigm of Th1 cell-mediated autoimmune disease, considered an animal model of MS. EAE can be induced in susceptible animals by active sensitization with CNS tissue, myelin or myelin proteins or by adoptive transference of autoreactive T cells [7,8]. Other similar features shared by EAE and MS are the genetic susceptibility and cytokine pattern displayed during the inflammatory immune response at the demyelinating lesions [9].

Cytokines have a key role in the EAE process providing the necessary signals to activate T cell specificity against self-antigens. There is overwhelming evidence to suggest that the T cells mediating EAE are of the Th1 phenotype, producing

Interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). Tissue expression of these cytokines at onset and acute phase of EAE support the idea of a disease-promoting role, whilst transforming growth factor-β (TGF-β) and IL-10 are related to the spontaneous recovery of EAE [10-13].

Gangliosides are a family of acidic glycosphingolipids present in all eukaryotic cells. Their molecular structure comprises a ceramide moiety as a lipophilic anchor embedded in the outer leaflet of plasma membranes and a sialo-oligosaccharide residue exposed towards the extracellular space [14] These compounds are ubiquitous constituents of cell membranes and have been implicated in a variety of biological events such as differentiation, proliferation, and morphogenesis [15]. In the immune system these glycosphingolipids have immunosuppressive properties, depending upon the structure, and concentration of gangliosides and nature of target and effector cells [16].

The aim of the present work was to investigate the mRNA expression, by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, of IL-2, IFN-γ, TNF-α, IL-10, and TGF-β in spleen cells from animals immunized with experimental autoimmune encephalomyelitis, to confirm the influence of gangliosides *in vitro* on the Th1/Th2 cytokines balance in EAE.

Results

Clinical score

To verify the effect of gangliosides on the progress of EAE, rats were sacrificed during neurological deficits and after recovery from clinical signs (Figure 1). The course of clinical signs was significantly ($p \leq 0.05$) suppressed in groups treated with gangliosides. In contrast, the disease was more severe in control groups. These results suggest a suppressor effect for the gangliosides in the progression of clinical signals.

Viability Assay

The effect of gangliosides on cellular viability were studied in spleen cells obtained from both groups of animals sacrificed during the neurological signs and after recovery The cells were maintained in culture for 48 and 72 hours under the stimulus of neuroantigen (GP-MBP), gangliosides. Control cells were not stimulated.

All cells (obtained from animals sacrificed during clinical signs and cultured for 48 h) , maintained with gangliosides *in vitro*, showed significantly lower viabilities ($p \leq 0.05$) than cells stimulated with GP-MBP or not stimulated (Figure 2A). At 72 hours of cell culture, this effect was observed only in cultures from animals treated with gangliosides (Figure 2B). Conversely, cells from animals sacrificed after recovery from disease and maintained for 48 and 72 hours, showed significant decreases in viability ($p \leq 0.05$) only in cell cultures obtained from animals that received gangliosides *in vivo* and *in vitro* (Figures. 3A and 3B).

Cytokine Expression by Spleen Cells

To analyze the modulation of gangliosides in spleen cells, we cultured cells from animals sacrificed during signs and after recovery from EAE. These cultures were

compared among the ganglioside treated, untreated and saline treated groups. Figure 4 depicts results obtained in cultures from animals sacrificed during clinical signs. The treatment with gangliosides, *in vivo,* modulates mRNA expression of Th1 cytokines, particularly IL-2, which presents significantly lower levels of Th1 cytokines than both control groups (untreated and saline treated). We observed that the levels of IFN-γ and TNF-α mRNA were reduced in spleen cells from the ganglioside-treated group, independently of culture stimulus. The addition of gangliosides to the culture medium, led to a reduction in mRNA expression of all proinflammatory cytokines evaluated. In contrast, cytokines involved with the recovery from clinical signs, such as TGF-β and IL-10, showed an increase in mRNA levels (Figure 5).

Figure 6 demonstrates Th1 cytokines mRNA levels in the cells of animals sacrificed after recovery of EAE. After recovery, the GP-MBP stimulated cultures from animals treated with gangliosides presented higher IFN-γ mRNA expression than those from the control groups (untreated or saline treated cultures). However, the cells from animals treated with gangliosides and cultured without stimulus exhibited significantly reduced IFN-γ mRNA levels ($p \leq 0.05$) in comparison with other groups. Spleen cells from animals treated with gangliosides, cultured with gangliosides and unstimulated, demonstrated reduced TNF-α mRNA levels when compared with spleen cells from control groups. Similar results were observed in the expression of TGF-β and IL-10 (Figure 7). In these cell cultures, the addition of gangliosides to the medium diminished mRNA levels of all cytokines evaluated.

Discussion

In the present work we studied the cytokine expression pattern of the spleen cells of animals sacrificed during two phases of EAE: a) during clinical signs and b) after recovery from the disease, in cell cultures maintained for 48 and 72 hours to evaluate the *in vitro* kinetics of the immunosuppressive effect of gangliosides in correlation with *in vivo* behavior.

The evaluation of clinical signs, confirms the action of exogenous ganglioside treatment on the evolution of EAE, since treated animals exhibit mild disease in contrast to animals from the control groups (untreated and saline-treated) in which the neurological deficit was considerably more severe.

Suppression of clinical signs of EAE by the gangliosides has been demonstrated in several studies using different protocols of disease induction. These studies show that the effects of gangliosides are achieved with high doses (100mg/kg) administered during the effector phase, presenting no effect if administered during inoculation or the sensitizing phase [17-19]. Furthermore, an attempt to induce EAE with gangliosides was demonstrated to be ineffective [20].

Immunosuppressive effects of gangliosides were observed *in vitro*, since spleen cells from animals treated with gangliosides, *in vivo*, and sacrificed during clinical signs showed diminished viability when compared with spleen cells from control groups (untreated and saline-treated), independently of the time of cell culture and the stimulus used.

Moreover, the addition of gangliosides to the cell culture medium significantly reduces the viability during the first 48 hours. However, in the cell cultures maintained for 72

hours, this reduction was observed only in spleen cells from animals treated with gangliosides.

In addition, we did not find any differences between the *in vitro* stimuli of spleen cells from animals sacrificed after EAE recovery. The significant decrease in viability was obtained only in spleen cells from animals treated *in vivo* and *in vitro* with gangliosides. These results suggest that cell viability can be affected by the administration of gangliosides *in vivo* and *in vitro*. Thus, spleen cells from animals treated with gangliosides remain susceptible to these compounds even when maintained in long-term cell culture (48 and 72 hours). This reduction in viability may be associated with cell death, demonstrated by apoptosis or necrosis, induced by the inhibition of nuclear localization of nuclear factor-κB (NF-κB). In this case, the cell death occasioned by the suppression of the NF-κB-dependent survival pathway suggests immunocompetent cell depletion due to an immunosuppression mechanism exercised by the gangliosides [21,22].

Studies using exogenous biotin-monosialoganglioside (biotin-GM-1) showed intracellular distribution along the endocytic pathway, suggesting that glycosphingolipids may become components in late endosome and lysosome membranes before their degradation. Conversely, gangliosides are rapidly incorporated into plasma membranes and concentrated in caveolae, where GM1 is believed to participate in the sphingolipid-cholesterol microdomains or rafts. A similar distribution has been reported in endogenous gangliosides [23-25]. In the plasma membrane, the microdomains, or rafts, can bind to the cytoskeleton proteins and phospholylated phosphotyrosines, acting as a transducing center for cellular signals [26, 27].

The results obtained in our viability assay correlate with the expression of cytokines, since a ganglioside action was observed in the immune response in treated animals and also after the addition of these compounds to the culture medium.

The Th1 cytokines, IL-2, TNF-α and IFN-γ are closely related to genesis and installation of the EAE. In the present work, spleen cells of animals sacrificed during clinical signs of the disease and treated with gangliosides showed significant cytokine mRNA suppression. In addition, the levels of mRNA expression of IL-10 and TGF-β were higher than those of control groups. These cytokines are related to spontaneous recovery from EAE.

However, there was a disagreement between these data and that previously obtained by Kanda and Watanabe. These authors showed that the individual gangliosides GD1b, GT1b, and GQ1b selectively enhance the Th1 cytokine production and decrease the Th2 expression [28]. The difference could be explained first, by the methodology used, these authors submitted the T cells from healthy donors to culture under nanomolar concentration of ganglioside (100nM) stimulus and second by the dose-dependent effects of these compounds on the immune system. The suppressive properties of gangliosides is evident at high concentrations (approximately 50μM, similar to the concentration found in CNS) [29,30].

Spleen cells from control groups (untreated and saline-treated), submitted to cell culture supplemented with gangliosides, exhibited a remarkable decrease in expression of almost all cytokines. However, in spleen cells obtained from animals sacrificed after recovery from EAE and kept under the same cell culture conditions, the effects of the gangliosides are enough to reduce cytokine expression. Such

results suggest that the time and dose-dependent properties of gangliosides act (i) specifically on autoreactive lymphocytes, since in spleen cells from recovered animals, the ganglioside effects are diminished, probably due to the reduction in these cell populations as a consequence of cell death, and (ii) at a transcriptional level. Some authors demonstrate specific inhibition, at a transcriptional level, of Th1 cytokines (IL-2, IFN-g and TNF) by gangliosides, without inhibition of Th2 cytokines (IL-4 and IL-10) [31-35]. Such an occurrence is explained by the inhibition of phosphorylation and I-κB degradation, the regulatory protein that binds to NF-κB, hindering its translocation to the nucleus, and consequently inhibiting cytokine mRNA transcription, promoting cell cycle arrest [31,32].

In contrast, the participation of exogenous gangliosides in CD4 molecule internalization is well known to modulate the immune response. This mechanism is related to the disorganization of the trimolecular complex in T cells, acting as a metabolic "off " in cell signaling [36-38].

The polarization of $CD4^+$ T cells populations for Th1 or Th2/Th3 plays a crucial role in EAE induction and regulation. The onset of disease is related to the increase in effector mechanisms of CD4+ Th1 T cells that express pro-inflammatory cytokines. Conversely, spontaneous remission is associated with the negative regulation of the inflammatory process by changes in the immune response, predominantly Th2/Th3 [39].

The results presented herein may represent important clues for further understanding of the action of gangliosides in the immune system, mainly in autoimmune disease, highlighting their potential role as immunomodulatory compounds.

Materials and Methods

Animals, inoculation and assessment of clinical signs

Female Lewis rats, aged 8 weeks, obtained from CEMIB-UNICAMP, were fed standard chow and had access to water *ad libitum*. Each rat was inoculated with 50μg of guinea pig myelin basic protein (GP-MBP, purified by the modified procedure of Deibler *et al.* [40]) dissolved in phosphate buffered saline (PBS, 0.1M, pH 7.4) and emulsified in complete Freund's adjuvant (CFA, Difco – Detroit — Mt, USA) supplemented with 300μg of *Mycobacterium tuberculosis* (H37RA – Difco). The animals were inoculated in the hind footpads (Table 1). Rats were examined daily for neurological signs and were scored as: 0, healthy; 1, limp tail; 2, hind limp paralysis; 3, hind limb paraplegia and incontinence; 4, quadriplegia and 5, moribund and dead. Procedures involving animals and their care were conducted in conformity with international laws and policies.

Administration of gangliosides

A mixture of purified gangliosides from bovine brain containing 21% GM1, 40% GD1a, 16% GD1b, and 19% GT1b (TRB-Pharma, Campinas-Brazil) was injected subcutaneously at a dose of 100mg/kg (3mM/kg) every 12 h, from the 7th day post-inoculation (dpi) to the 15th dpi. Rats from the saline group received 0.9% saline solution at similar time intervals and underwent identical manipulation each day (Table 1).

Table 1. Experimental groups

Table 1. The experimental groups used in this study

Group	N	Sensitization	Treatment	Sacrificed
Untreated	3	GP-MBP+CFA+Mt	None	During clinical signs
Saline	3	GP-MBP+CFA+Mt	Saline	During clinical signs
Gangliosides	4	GP-MBP+CFA+Mt	Gangliosides	During clinical signs
Untreated	3	GP-MBP+CFA+Mt	None	After recovery
Saline	3	GP-MBP+CFA+Mt	Saline	After recovery
Gangliosides	3	GP-MBP+CFA+Mt	Gangliosides	After recovery

GP-MBP=guinea pig myelin basic protein; **CFA**=complete Freund's adjuvant; ***Mt***=*Mycobacterium tuberculosis*

Mononuclear spleen cell isolation

Spleen cells from groups sacrificed during clinical signs and after recovery from the disease were excised, sieved through a stainless-steel mesh screen (Sigma – San Diego-CA, USA) and washed three times in Hank's balanced salt solution (Sigma). Isolated spleen cells were suspended in RPMI 1640 supplemented with glutamine (HyClone – Logan-UT, USA) containing 10% fetal bovine serum (FBS – HyClone) 2-mercaptoethanol (1/5000 – Life Technologies - Grand Island-NY, USA), and 5μl/ml penicillin/streptomycin/ml (Life Technologies).

Viability Assay

Spleen cells ($2x10^6$ cells/ml) were cultured in 96 well flat bottom microculture plates in RPMI 1640 supplemented with glutamine (HyClone) containing 10% FBS, 2-mercaptoethanol (1:5000 – Life Technologies) and 5μl/ml penicillin/streptomycin (Life Technologies). Triplicate cultures were stimulated with 25μg/ml GP-MBP, 400μg/ml (50μM) mixture of gangliosides or maintained with complete medium alone, incubated for 48 and 72 hours in a humidified atmosphere of 5% CO_2 at 37°C. MTT tetrazolium [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] (5mg/ml stock solution – Sigma) was added to medium cultures (10μl/well) and incubated for 4h (5% CO2 at 37°C). Subsequently each well received 100μl 10% SDS in 0.01HCl and was incubated for 15 minutes. The plates were read on a microplate reader at 540 and 640 nm (SpectraMax 196, Molecular Devices, Sunnyvale, CA, USA). Results were expressed as cells/ml.

Reverse transcriptase-polymerase chain reaction analyses for cytokines

Total cellular RNA was isolated by a modified protocol of acid guanidium thiocyanate/phenol/chloroform extraction [41, 42]. RNA was first reverse-transcribed into cDNA (Superscript^{tm} II – Gibco). PCR amplification was perfomed in a total volume of 50μl containing 2μl cDNA, 200ng of each primer (Table 2), 500μM dNTP (Pharmacia Biotech – Piscataway-NJ, USA) and 1U Taq polymerase (Life Technologies) in a buffer supplied by the polymerase manufacturer. Optimum PCR conditions were determined for each set of primers as follows: IL-2, 40 cycles (60°C for 50s, 72°C for 90s, 95°C for 45s); IFN-γ, IL-10 and TGF-β, 40 cycles (58°C for 45s, 72°C for 90s, 95°C for 45s); TNF-α, 40 cycles (57°C 50s, 72°C for 90s, 95°C for 45s). Sample contamination by genomic DNA was verified by submitting the RNA sample to PCR amplification omitting the RT step. The PCR products were submitted to 1.5% agarose gel electrophoresis containing ethidium bromide and visualized by excitation under ultraviolet light and digitally recorded using the Nucleovision® system (NucleoTech, San Mateo, CA, USA) and their molecular weight and band pixel area of PCR products were calculated using the Gel Expert® Software (NucleoTech). The Semiquantitative Expression (SE) of cytokines was calculated for each sample using the following formula and expressed as arbitrary units (AU): SE = pixel area of the product to be analyzed/pixel area of cyclophilin x 100.

Statistical analysis

Comparisons of results were analyzed using the Kruskal-Wallis test (non-parametric ANOVA). Values of $p \leq 0.05$ were considered as indicative of significance.

Table 2. Primers used for RT-PCR

Primer	Sense	Anti-sense	BP
IL-2	5'CATGTACAGCATGCAGCTCGCATCC 3'	5'CCACCACAGTTGCTGGCTCATCATC 3'	410
IFN-γ	5'GTTTTGCAGCTCTGCCTCATGGCCCTCT 3'	5'CAGCACCGACTCCTTTTCCGCTTCCTTA 3'	446
TNF-α	5'GTGCCTCAGCCTCTTCTCATTCC 3'	5'GCTCCTCCGCTTGGTGGTTT 3'	218
IL-10	5'CTGCTATGTTGCCTGCTCTTAC 3'	5'TCATTCTTCACCTGCTCCACT 3'	419
TGF-β	5'CCGCAACAACGCAATCTATG 3'	5'GCCCTGTATTCCGTCTCCTT 3'	304
Cyclophilin	5'GACAGCAGAAAACTTTCGTGC 3'	5'GGTTCTGACTCACCGACCT 3'	276

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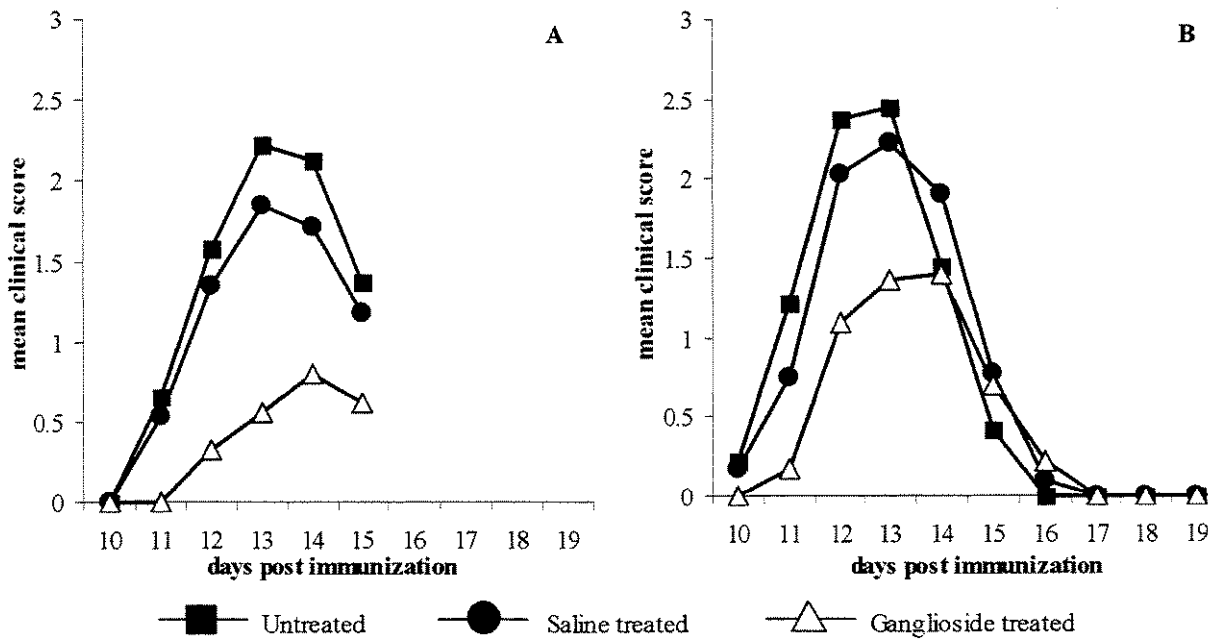


Figure 1. Mean clinical score of rats sacrificed during clinical signs (A) and after recovery (B) from EAE. The rats from the control groups (untreated and treated with saline) demonstrated more severe neurological deficits than rats in the ganglioside-treated group.

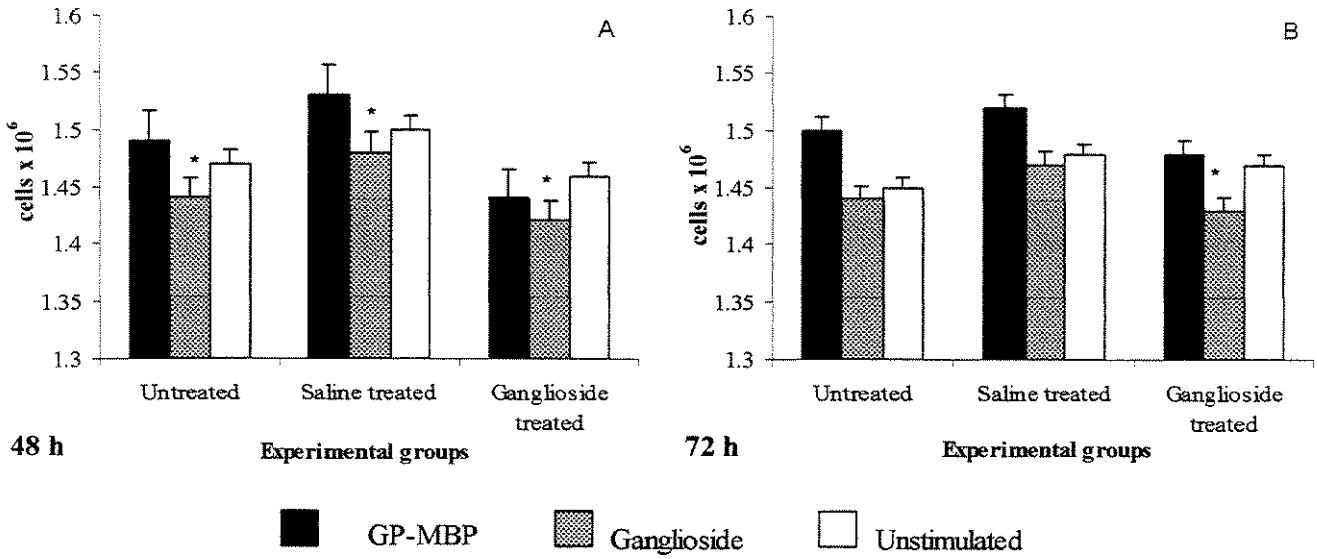


Figure 2. Viability assay. Spleen cells were isolated from animals sacrificed during clinical signs of EAE and maintained for 48 and 72 hours in medium enriched with GP-MBP, gangliosides, or unstimulated. A significant decrease in the viability of cells cultured under ganglioside stimulus, was observed independently of experimental group (A). After 72 hours the effect of the gangliosides on viability persisted only in the spleen cells from animals treated previously with gangliosides (B). Values represent means ± SEM of triplicate cell cultures. Data represent three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$ comparing *in vitro* treatments by the Kruskal-Wallis test (non parametric ANOVA).

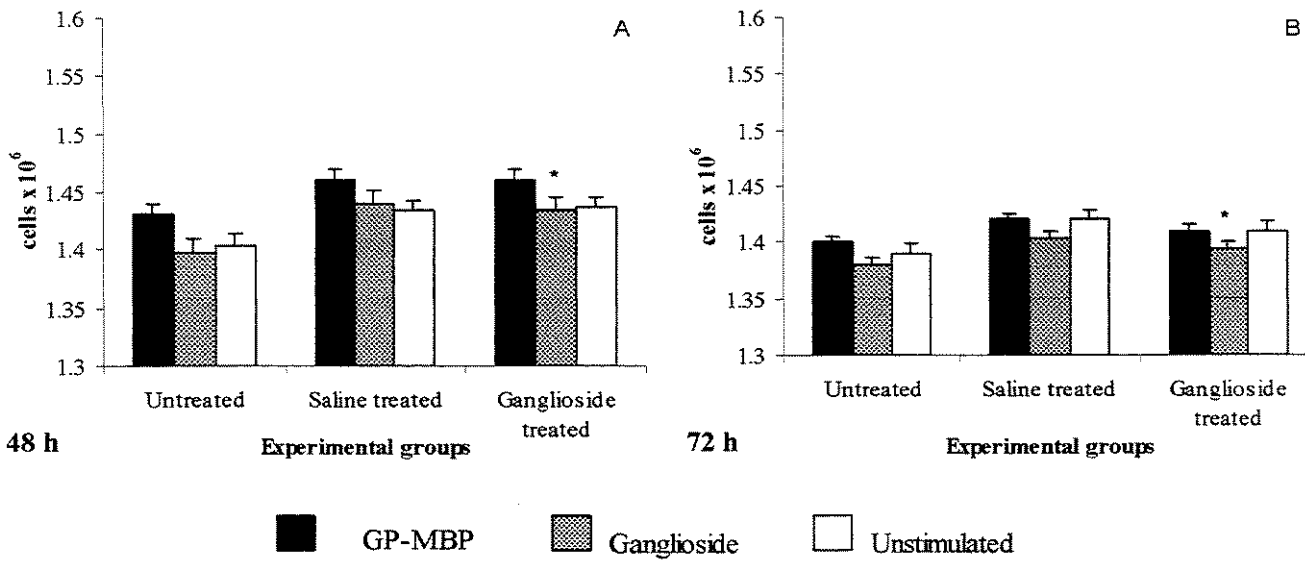
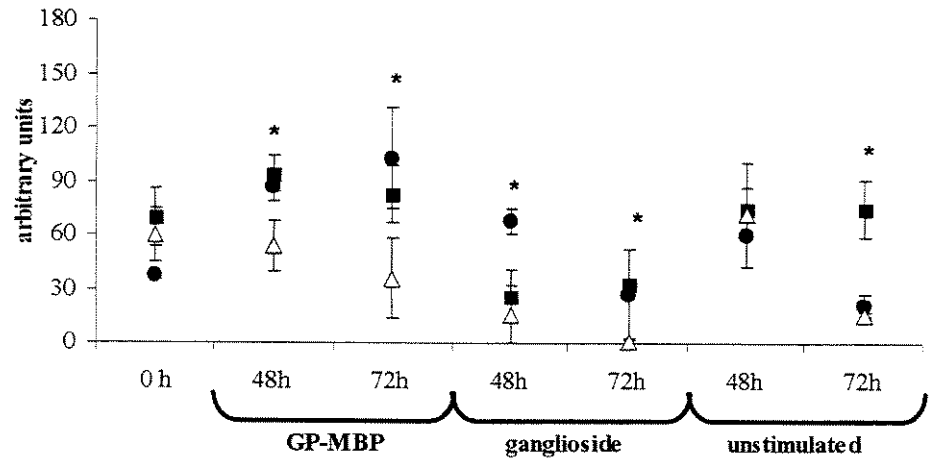
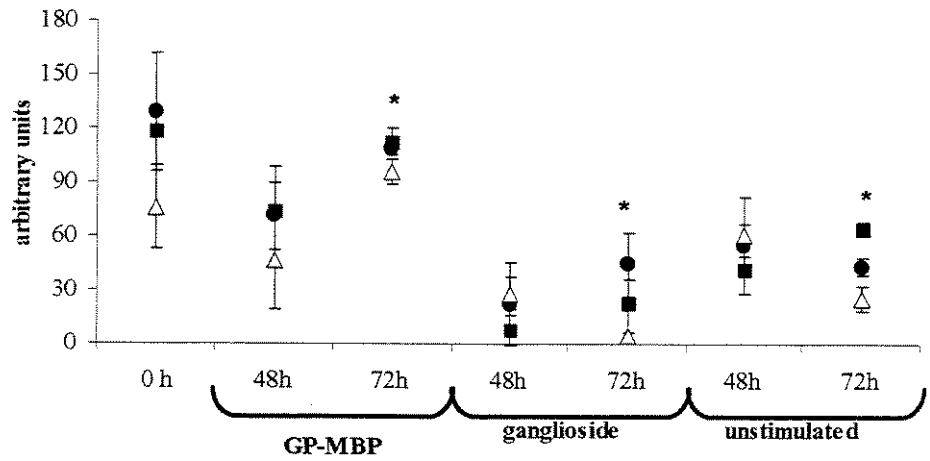


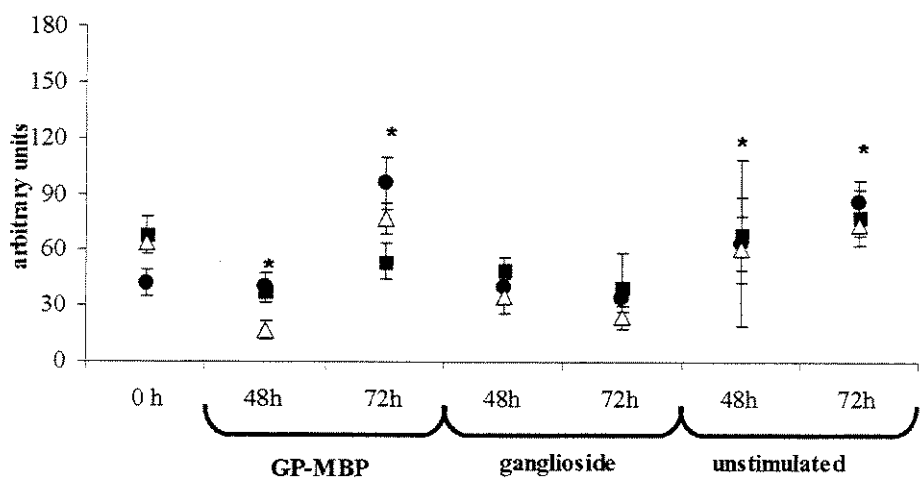
Figure 3. Viability assay of spleen cells isolated from animals sacrificed after recovery from EAE. The cell cultures were maintained for 48 and 72 hours in medium under stimulus with neuroantigen (GP-MBP) and enriched with gangliosides. Although all cell cultures maintained in the presence of gangliosides exhibited a decrease in cell viability, statistical significance was observed only in cell cultures from animals previously treated with gangliosides (A). After 72 hours, the same effect of the gangliosides on the viability was observed (B). Values represent the means ± SEM of triplicate cell cultures. Data represent three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$, comparing treatments *in vitro* by the Kruskal-Wallis test (nonparametric ANOVA).



IL-2 ***in vitro*** **stimuli**



INF-γ ***in vitro*** **stimuli**



TNF-a ***in vitro*** **stimuli**

Figure 4. Expression of mRNA cytokines in spleen cells cultured at 0, 48 and 72h. Cells from animals sacrificed during clinical signs of EAE. Cells obtained from animals treated *in vivo* with gangliosides exhibited a decrease in mRNA expression of proinflammatory cytokines, even when stimulated with neuroantigen (GP-MBP) or unstimulated. Cell cultured with gangliosides demonstrated decreased mRNA expression of IL-2, IFN-gamma, and TNF-alpha. Values are means $\pm$ SEM of triplicate cultures. Data represent three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$, comparing treatments *in vitro* by the Kruskal-Wallis test (nonparametric ANOVA).

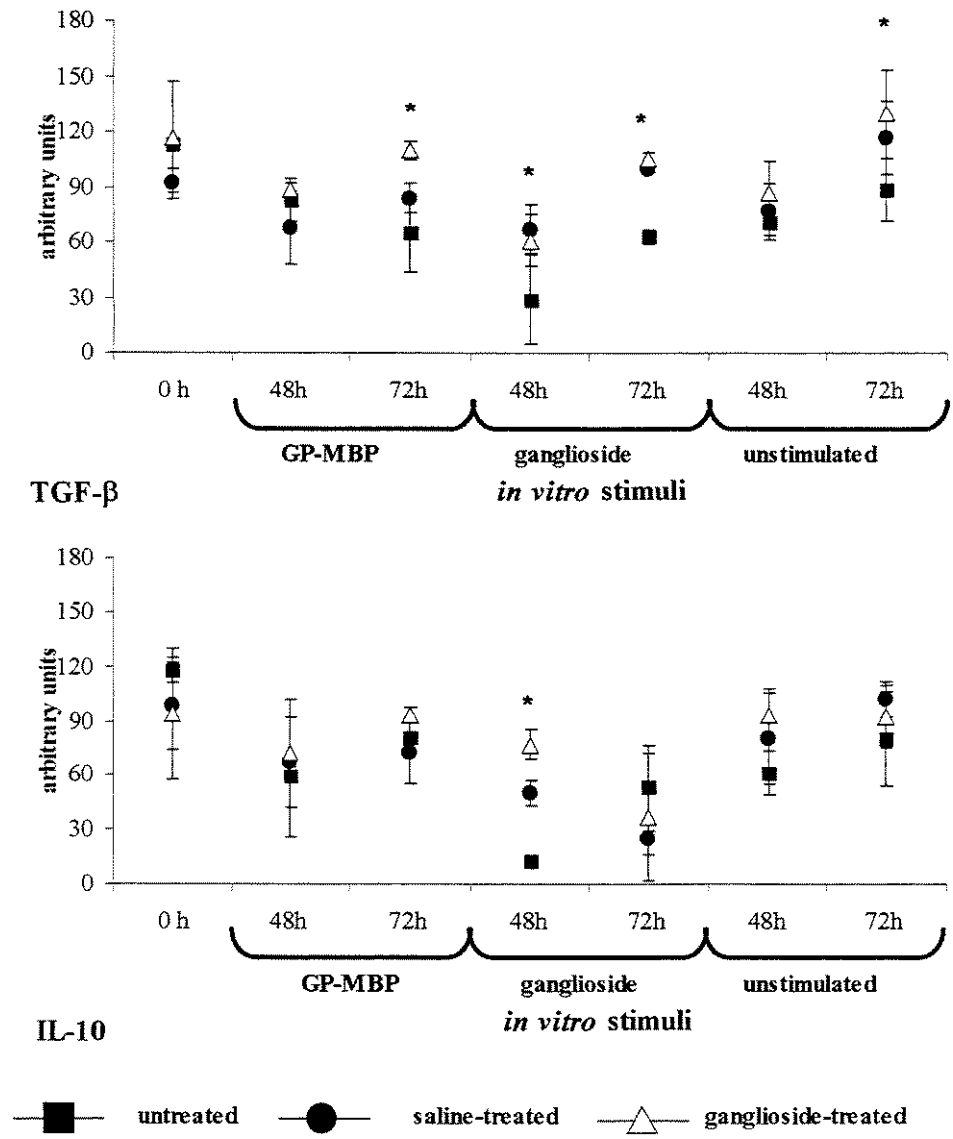

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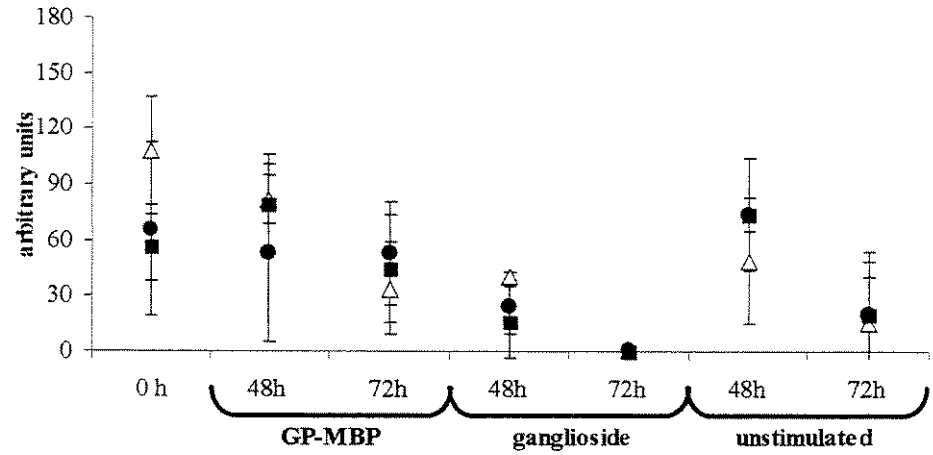
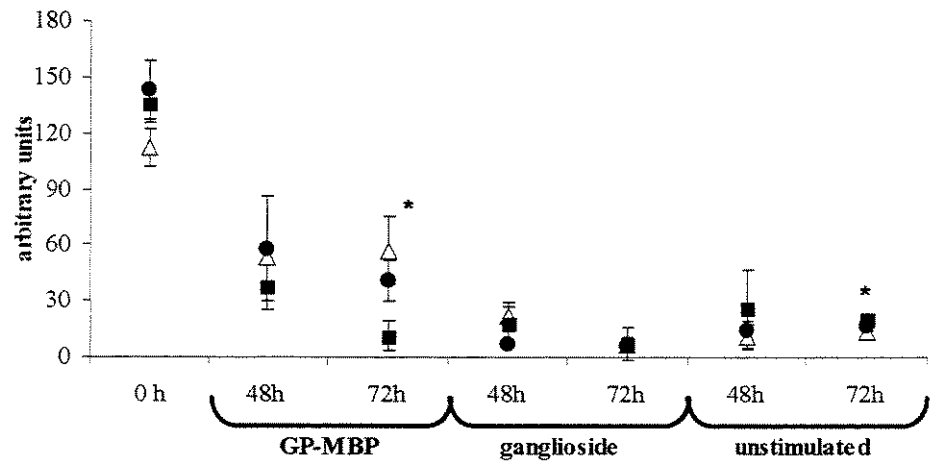
Figure 5. Expression of cytokine mRNA in spleen cells cultured at 0, 48 and 72h. Cells were isolated from animals sacrificed during clinical signs of EAE. The mRNA of TGF-β and IL-10, antiinflammatory cytokines involved in recovery, was increased in the cultures of cells from animals treated with gangliosides. Values are means ± SEM of triplicate cultures. Data represent three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$ compared with *in vitro* treatments by Kruskal-Wallis test (nonparametric ANOVA).

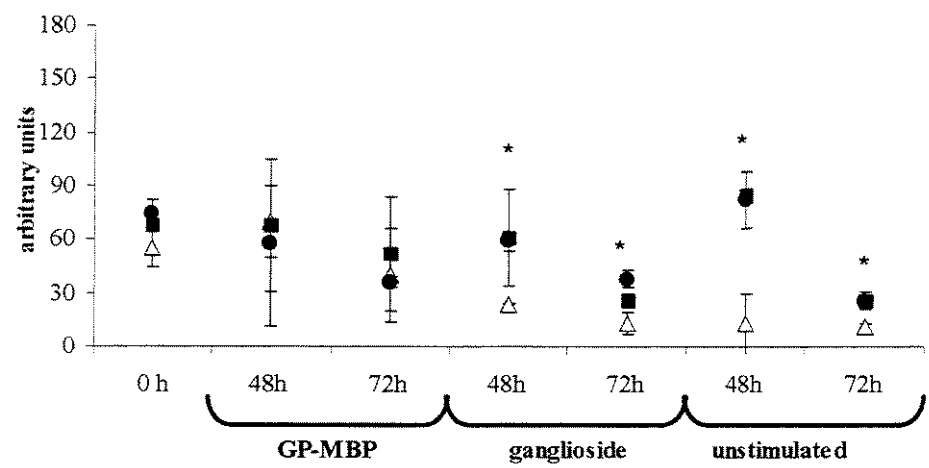
Figure 6



IL-2 *in vitro* stimuli



IFN-γ *in vitro* stimuli

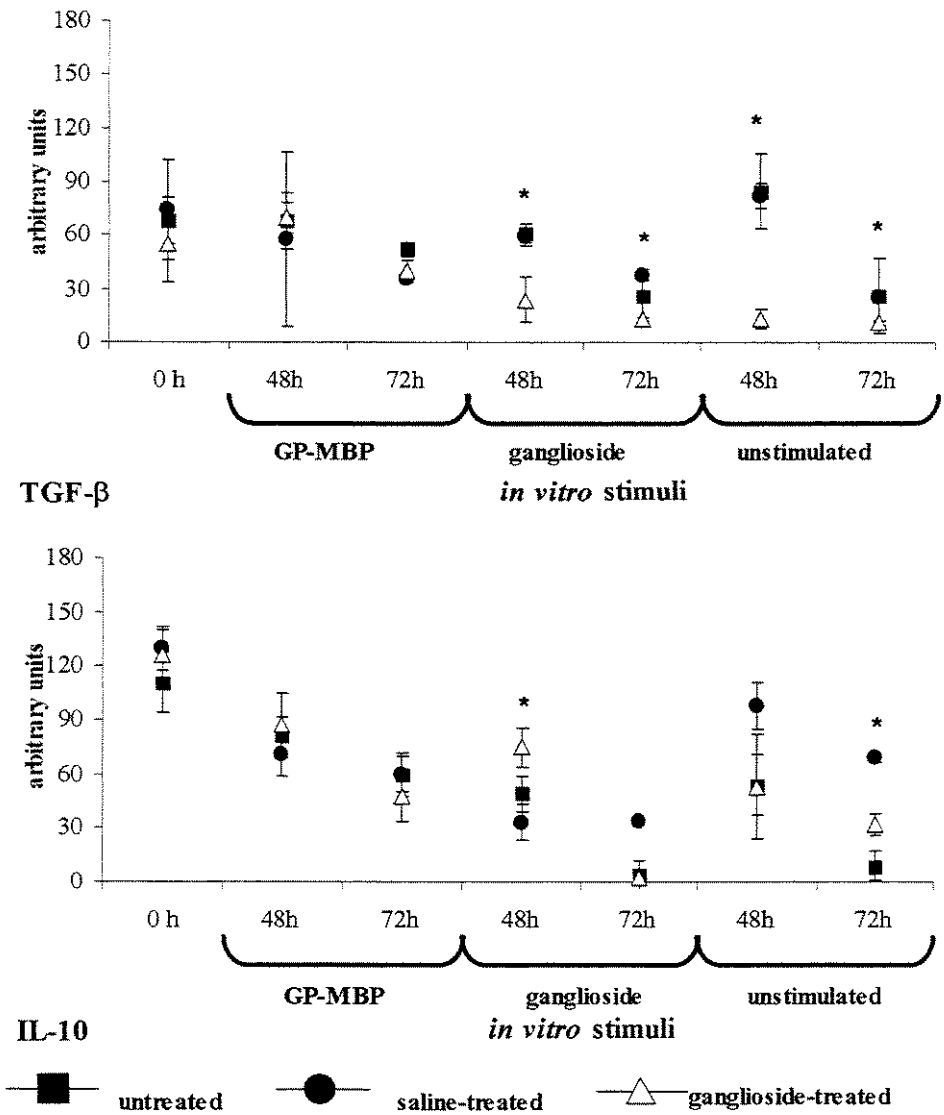


TNF-α *in vitro* stimuli

untreated saline-treated ganglioside-treated

Figure 6. Expression of TH1cytokines mRNA in spleen cells cultured for 0, 48 and 72h. Cells were isolated from animals sacrificed after recovery from EAE. After recovery, the pattern of cytokine expression demonstrated that spleen cells from all experimental groups, maintained in the presence of neuroantigen, exhibit a similar behavior. The addition of gangliosides to the medium led to decreases in all cytokine levels, with the exception of IL-2 in the spleen cells from the saline-treated group, which demonstrated higher levels than those of spleen cells from both untreated and ganglioside-treated groups. Values are means $\pm$ SEM of triplicate cultures. Data represent three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$, comparing treatments *in vitro* by the Kruskal-Wallis test (nonparametric ANOVA).

Figure 7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untreated
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ganglioside-treated

Figure 7. Expression of TH2/TH3 cytokine mRNA in spleen cells cultured for 0, 48 and 72h. Cells were isolated from animals sacrificed after recovery from EAE. Although the pattern of cytokine expression is similar at all times, cells from animals treated with gangliosides and maintained with these compounds, *in vitro,* showed a significant increase in IL-10 mRNA expression. Values are means ± SEM of triplicate cultures. Data represent three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$, comparing treatments *in vitro* by the Kruskal-Wallis test (nonparametric ANOVA).

CYTOKINES RELATED TO LIFE AND DEATH STIMULI: EFFECT OF GANGLIOSIDE ON GENE EXPRESSION OF ANTI AND PRO-APOPTOTIC MOLECULES IN EAE

Objetivos:

1. Investigar a ação do tratamento com gangliosídeos sobre a manifestação das deficiências neurológicas da EAE.
2. Avaliar a expressão de citocinas relacionadas com estímulo de sobrevivência e morte no sistema nervoso central e células esplênicas, durante a evolução da EAE.
3. Determinar a expressão do mRNA de proteínas relacionadas com a apoptose bem sua ocorrência nos animais tratados com gangliosídeos e seus respectivos controles, em três fases distintas da EAE: anterior aos sinais clínicos, durante os sinais clínicos e após a recuperação.

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CYTOKINES RELATED TO LIFE AND DEATH STIMULI: EFFECT OF GANGLIOSIDE ON GENE EXPRESSION OF ANTI AND PRO-APOPTOTIC MOLECULES IN EAE

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RUNNING TITLE: Life and death stimuli in EAE under ganglioside effect

Key words: IL-12/TGF-β/EAE/Gangliosides/Bcl-2 /apoptosis/Fas – FasL

Submitted

Abstract

IL-12 and TGF-β are antagonist cytokines acting in the genesis and recovery of EAE, respectively. Besides their modulatory properties, these cytokines can modulate apoptosis molecules. IL-12 can rescue lymphocytes from the cell death program by induction of anti-apoptotic molecules, such as the BCL-2 family proteins. In the same way, TGF-β induces the imbalance of the anti-apoptotic and pro-apoptotic signals, thus initiating the cell death program. In the present study, we used the model of acute EAE to evaluate IL-12 and TGF-β expression in relation to the expression of anti-apoptotic and pro-apoptotic molecules under exogenous ganglioside administration. Our data provide evidence that the ganglioside action on the *in vivo* Bcl-2, Bcl-W, Fas, and FasL expression is associated with cytokine modulation. Results demonstrate that ganglioside treatment leads to an increase in TGF-β levels in brain and spleen cells, together with an increase in Fas and FasL mRNA expression. In contrast, IL-12, BCL-2 and BCL-W presented reduced levels in the ganglioside-treated group. These results suggest that the immunomodulatory action orchestrated by the gangliosides, act upon the survival signals to down-regulate anti-apoptotic Bcl-2 family expression and up-regulate TGF-β expression, together with an increase in Fas and FasL expression, consequently suppressing the immune response.

Introduction

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are central nervous system (CNS) inflammatory diseases characterized by the accumulation of $CD4^+$ T and mononuclear cells that lead to myelin damage. Cytokines play a crucial role in this process by regulating aberrant autoimmune responses against the CNS by promoting or limiting disease [1].

In this context, interleukin-12 (IL-12), produced by antigen-presenting cells (APCs), is necessary for T helper 1 (Th1) response development leading to an increase in pro-inflammatory cytokines production (mainly IFN-γ). In EAE, IL-12 is crucial early in the development of the disease, inducing differentiation of the precursor T cells into the Th1 phenotype [2]. Furthermore, increases in IL-12 levels have been reported in the serum of patients with MS in clinically active disease stages [3,4].

The transforming growth factor-β (TGF-β) exerts an inhibitory effect on the immune response, limiting the autoagressive T cell response by preventing APCs maturation [5,6]. Moreover, TGF-β is involved in the spontaneous recovery of the Lewis rat from EAE, and in the oral tolerization to MBP (myelin basic protein), which suppressor cells that secret TGF-β inhibit IFN-γ production [7].

IL-12 and TGF-β are antagonistic cytokines with an essential role in multiple sclerosis and experimental autoimmune encephalomyelitis pathogenesis and recovery, respectively. These cytokines are also key regulators of T helper (Th)1/Th2 balance and disruption of this system has been shown to lead to autoimmunity [8].

Besides their immunomodulatory effects, IL-12 and TGF-β can modulate apoptosis via the survival or death signals. IL-12 can inhibit apoptosis of $CD4^+$ T cells by

activation-induced or Fas-mediated apoptosis, down regulating FasL [9]. Conversely, TGF-β treatment reduces concomitant Bcl-2 expression by increasing Bax expression, rendering cells susceptible to apoptosis [10].

In acute experimental autoimmune encephalomyelitis (EAE), the animal model for studying the pathogenesis of multiple sclerosis, autoreactive T cells are eliminated from the CNS by activation-induced cell death (AICD), involving the Fas pathway [11, 12].

In contrast, the cell expression of anti-apoptotic members of the Bcl-2 family protein confer relative protection against apoptosis [12]. Bcl-xL transgenic mice with EAE, induced by myelin oligodendrocyte glycoprotein (MOG), have an earlier onset and more severe form of EAE when compared with wild-type C57BL/6 mice and nontransgenic littermates [13].

Gangliosides are complex glycosphingolipids that contain a hydrophilic head group with one or more sialic acid residues and a hydrophobic tail group of ceramide [14]. Many reports provide evidence that these compounds have immunosuppressive properties such as CD4 down-modulation [15,16], cell cycle arrest of EL4 cells [17], and inhibition of the production of the Th1-associated cytokines without blocking the production of the Th2-associated cytokines [18].

In addition, gangliosides can induce T cell apoptosis in a dose and time-dependent manner [19]. It has also been demonstrated that cell death, mediated by the GD3 ganglioside, involves two branches: the Fas pathway [20] and the induction of cytochrome *c* release and mitochondrial permeability transition [21].

In the present study, the effect of the administration of gangliosides to Lewis rats with EAE on IL-12 and TGF-β expression and the modulation of apoptosis-related molecules expression and cell death induction is investigated. Our results suggest that ganglioside-induced apoptosis participates in the immunomodulation, exerted by these compounds, on IL-12 down-modulation and TGF-β up-modulation, acting to suppress the expression of the survival factors, Bcl-2 and Bcl-W, and stimulating death factors, Fas and FasL.

Results

Gangliosides protect Lewis rats from EAE

The clinical signs of EAE demonstrated that the animals from control group developed typical disease with severe neurological deficits (figure 1). The ganglioside-treated group began one day after the onset of clinical signs in the control group, and the severity of disease, was significantly milder($p=0.0067$).

Cytokines related to life and death stimulus

We evaluated IL-12 expression, which plays an essential role in the pathogenesis of EAE and MS [22], and TGF-β, a cytokine with a potent immunosuppressive property [23].

In the spinal cord, IL-12 expression was significantly ($p=0.0093$) lower than in the control groups during EAE signs. With EAE resolution, all experimental groups exhibited a decrease in IL-12 mRNA levels (Figure 2A), and ganglioside-treated groups had significantly lower levels ($p=0.05$) than those of the control group. The spleen cells presented similar levels during clinical signs in all experimental groups. After recovery, the IL-12 expression observed in the control group was maintained, in contrast to the ganglioside-treated group in which IL-12 levels were significantly lower ($p= 0.05$) (figure 2B).

The analysis of TGF-β mRNA expression in the spinal cord, during clinical signs, demonstrated that animals treated with gangliosides presented significantly higher levels ($p=0.0085$) in comparison to the control group (figure 3A), declining after recovery. A similar pattern was observed in the spleen cells the ganglioside-treated

group exhibited significantly higher levels both during clinical signs (p=0.03) and after recovery (p=0.03) than the control group (figure 3B).

Expression of Apoptosis-Related Molecules

To verify whether the gangliosides could suppress the clinical signs by apoptosis induction, we analyzed the expression of Fas, FasL, Bcl-2, and Bcl-W mRNA in the spinal cord and spleen cells from animals inoculated with GP-MBP.

The assessment of the expression of two anti-apoptotic members of the Bcl-2 family proteins in the spinal cord during clinical signs in ganglioside-treated animals, demonstrated significantly lower Bcl-2 levels (p=0.0254) than the saline-treated group (figure 4A). In the same way, during clinical signs, the spleen cells from the ganglioside group presented significantly lower levels (p= 0.01) than the saline treated animals. However, following recovery, the Bcl-2 expression of the saline group exhibited significantly lower levels (p=0.0003) than the ganglioside treated animals (figure 4B).

The Bcl-w expression behaved similarly to that of BCL-2, it was significantly reduced in ganglioside treated animals during clinical signs (p=0.02) and after recovery (p=0.0032), compared to the control group (figure 4C). Conversely, during clinical signs, Bcl-W levels were significantly higher (p= 0.028) in spleen cells from the gangliosides group. After recovery from EAE, the mRNA expression was similar in both experimental groups (figure 4D).

Regardless of differences observed in the expression of Bcl-2 and Bcl-W between treated and untreated groups, it should be pointed out that the expression of these molecules in the ganglioside-treated group remained unaltered during the entire EAE.

In addition to the anti-apoptotic Bcl-2 family members, we investigated the Fas and FasL gene expression, suggested to play an important role in the elimination of autoreactive T cells in EAE recovery [24]. The mRNA levels of Fas in the spinal cord from the gangliosides group were significantly higher than those of the control group, observed both during clinical signs or after recovery ($p=0.03$) (figure 5A).

Similarly to the spinal cord, spleen cells from the ganglioside-treated animals exhibited significantly higher Fas mRNA levels ($p=0.0286$) than those of the control group during neurological signs of EAE. After recovery, the Fas levels remained at the same level in the ganglioside-treated group, in contrast to the saline-treated group, which presented an increase in Fas mRNA expression (figure 5B).

The FasL expression in the spinal cord was significantly higher in the ganglioside-treated group ($p=0.0091$) during clinical signs and after recovery ($p=0.0016$). No significant alteration was observed in the animals from the saline-treated group during the clinical course of EAE (figure 5C). Similarly to the findings in spinal cord, the FasL mRNA expression in the spleen cells of the ganglioside-treated group, during the clinical signs of EAE, exhibited significantly higher levels ($p= 0.03$) than those of the control group. After EAE recovery, the FasL expression of spleen cells from animals treated with saline was significantly higher than that of the control group ($p=0.0286$) (figure 5D).

To verify the occurrence of apoptosis, *in situ* TUNEL assays were performed on spinal cord samples taken during clinical signs of EAE. The number of apoptotic cells in the saline-treated group (figure 6a and 6b) was substantially decreased when compared with the ganglioside-treated group (figure 6c and 6d).

Discussion

The aim of this study was to characterize the mRNA expression, *in vivo,* of IL-12 and TGF-β in correlation with the apoptosis-related molecules; Bcl-2 and Bcl-W survival factors and Fas/FasL, in animals inoculated with GP-MBP and treated with gangliosides. This is the first report to suggest, in the EAE model, that the gangliosides may modulate the gene expression of apoptosis-related molecules in the CNS and spleen cells.

Our results reinforce the evidence for the immunomodulatory action of gangliosides in EAE evolution, since the animals treated with gangliosides exhibited milder neurological signs and their duration in these animals was shorter than in the control animals. This immunosuppressive ganglioside effect has been observed in chronic relapsing-remitting EAE in NOD mice model [25], acute monophasic EAE [26], and Theiler's murine encephalomyelitis [27]. The daily administration of gangliosides significantly suppresses neurological manifestation and histological lesions in a dose-dependent manner [27].

In addition to the clinical observation, we observed that IL-12 levels were decreased in gangliosides treated animals. This finding, associated with the late initiation of EAE observed in these animals, suggests that gangliosides have an effect upon the genesis of the disease.

Experiments with IL-12 *p35-/- and p40-/-* mice demonstrated that this cytokine was required for Th1 differentiation of IFN-γ-producing T cells during a primary immune response, and that IL-12$^{-/-}$ mice are resistant to EAE induction [28,29]. IL-12 is mainly produced by monocytes and dendritic cells and gangliosides can inhibit the

development and function of these cells by interfering in the signaling pathways affecting the cytoskeleton, the traffic of membranes endocytosis, antigen migration and presentation, consequently inhibiting the synthesis of proinflammatory cytokines, particularly IL-12 [30,31].

In parallel to the decrease in IL-12 expression, our results demonstrate an increase in TGF-β expression in animals treated with gangliosides. TGF-β reduces the relapse severity of chronic EAE, protects the animals from disease signs, and causes the deactivation of macrophages and microglia. Conversely, the disruption of the TGF-β gene leads to massive inflammation, lymphocytic infiltration and spontaneous activation of T cells. In addition, regulatory cells TGF-β producers are associated with acute EAE recovery, as well as with the induction of oral tolerance [32-35]. Moreover, in our laboratory the addition of gangliosides to the culture medium of spleen cells from animals with EAE, was shown to stimulate TGF-β expression (Castro *et al.*, submitted).

IL-12 can suppress apoptotic cell death via the up-regulation of Bcl-2, this survival pathway activation occurs via NF-κB nuclear translocation, preventing death receptor-mediated apoptosis and down-regulating the expression of FasL [36,37].

In contrast, TGF-β can induce apoptosis in T cells by shifting the intracellular balance between death-promoting and death-inhibiting factors towards death induction. This process is not well understood, but the involvement of caspase 1-like protease and the cleavage of Bcl-X_L, leading to cell death, has been suggested as well as the up-regulation of pro-apoptotic Bax and the down-regulation of anti-apoptotic Bcl-2

[10,38,39]. Ours results are in agreement with the findings of these studies which relate the cytokines to life and death stimuli.

In EAE, clinical remissions and recovery from clinical symptoms may be related to inflammatory cell death through apoptosis, highlighting the crucial role of T-cell apoptosis in the outcome of an autoimmune attack and indicating that the termination of a T cell-mediated autoimmune reaction is dependent upon clearance of autoreactive cells through programmed cell death [13,40].

Although the effects of gangliosides on apoptosis have been extensively studied, the action of these compounds in EAE recovery in relation to cell death has not yet been studied *in vivo*. The TUNEL procedure demonstrated that ganglioside treated animals present a substantially higher number of apoptotic cells than the saline-treated group. These cells are localized mainly near to the blood vessel, suggesting their participation in the inflammatory infiltrate. However, even without the immunohistochemistry analysis for identification of these cells, their location and the clinical score of the treated animals allow us to infer their lymphocytic origin.

These results, together with the evaluation of the expression of apoptosis-related molecules demonstrating the upregulation of Fas and FasL concomitant with a decrease in the expression of the survival factors Bcl-2 and Bcl-W, suggest that gangliosides may initiate the cell death program.

Evidence of NF-κB activation on EAE pathogenesis exists, since its blockage leads to the inhibition of the induction of proinflammatory molecules [41]. This inhibition occurs at a transcriptional level since the generation of specific mRNAs encoding these cytokines is prevented [18].

In addition to proinflammatory cytokines induction, the transcription factor NF-κB is known to activate survival pathways and our results suggest that gangliosides have a role in apoptosis, acting as repressors of NF-κB complexes translocation to the nucleus and blocking the induction of survival genes [42].

The low Bcl-2 mRNA level in spinal cord during clinical signs in ganglioside treated animals suggests that these compounds can affect the cytochrome *c* pathway, in turn, down-modulating survival factors, as shown by Péguet-Navarro et al. [43] using GM3 and GD3 melanoma-derived gangliosides. Furthermore, many gangliosides from the a-series (GD3 and GD1a) and b-series (GD1b, GT1b, and GQ1b) may induce cytochrome *c* release from isolated mitochondria, rendering the cells susceptible to programmed cell death, in the absence of apoptotic signals [44]. After release, cytochrome *c* forms a complex with Apaf 1, dATP, and procaspase 9, resulting in caspase 9 activation, which activates the effector caspase 3 [45].

Our findings demonstrate pathways in which gangliosides can exert their immunomodulatory properties, stimulating TGF-β expression and, in consequence, promoting cell death by the Fas/FasL system, or down-modulating IL-12 and survival factors, which also lead to apoptosis.

Materials and Methods

Animals, inoculation and assessment of clinical signs

Female Lewis rats, aged 8 weeks, obtained from CEMIB-UNICAMP, were fed standard chow and had access to water *ad libitum*. Each rat was inoculated with 50μg of guinea pig myelin basic protein (GP-MBP) purified by the modified procedure of Deibler *et al.* [46], which was dissolved in phosphate buffered saline (PBS, 0.1M, pH 7.4) and emulsified in complete Freund's adjuvant (CFA, Difco – Detroit — Mt, USA), supplemented with 300μg of *Mycobacterium tuberculosis* (H37RA – Difco). The animals were inoculated in the hind footpads. The rats were examined daily for neurological signs and were scored as: 0 healthy, 1 limp tail, 2 hind limp paralysis, 3 hind limb paraplegia and incontinence, 4 quadriplegia and 5 moribund and dead.

Administration of gangliosides

The ganglioside-treated group received a mixture of purified gangliosides from bovine brain, containing 21% GM1, 40% GD1a, 16% GD1b, and 19% GT1b (TRB-Pharma, Campinas-Brazil), injected subcutaneously at a dose of 100mg/kg every 12 h, from the 7^{th} day post-inoculation (dpi) to 15^{th} dpi. Rats from the saline group received 0.9% saline solution at similar time intervals and underwent identical manipulation each day.

Tissue specimens

The experimental rats (n=3 in each experimental group) were killed under anesthesia (sodium pentobarbital, 50 mg/kg), tissue samples were taken on days 7, 15 and 19 post-immunization (dpi) before, during and after recovery clinical signs of EAE, respectively, and their spinal cords were removed and stored at –70ºC until mRNA

expression analysis. For morphological analysis, the spinal cords were snap-frozen in liquid nitrogen. Spinal cord sections (10 μm thick) were obtained on a cryostat.

Detection of in situ apoptosis by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (TUNEL)

The samples, obtained from Lewis rats sacrificed at 15th dpi, were submitted to the TUNEL reaction carried out according to the manufacturer's recommendations (Boehringer Manheim GmbH, Mannheim, Germany). Briefly, sections were fixed in 4% paraformaldehyde for 30 minutes, and permeabilized for 2 minutes in a 0.1% Triton X-100 in a 0.1% sodium citrate solution. After blocking for endogenous peroxidase activity with 0.3% hydrogen peroxide/methanol, a TUNEL reaction mixture was applied for 60 minutes at 37ºC. An anti-fluorescein peroxidase-conjugated antibody (POD) solution was used to detect the incorporation of dUTP, and slides were developed using 3,3'diaminobenzidine (DAB, 0.6 mg/ml, Sigma) in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.03% H_2O_2. The sections were counterstained with Harris' haematoxylin and then treated for the saponification reaction with lithium carbonate. The slides were mounted with coverslips using Entelan (Merck).

Mononuclear spleen cells isolation

Spleen cells from groups sacrificed during clinical signs and after recovery of the disease were excised, sieved through a stainless-steel mesh screen (Sigma – San Diego-CA, USA) and washed three times in Hank's balanced salt solution (Sigma). Isolated spleen cells were suspended in RPMI 1640 supplemented with glutamine (HyClone – Logan-UT, USA) containing 10% fetal bovine serum (FBS – HyClone) 2-

mercaptoethanol (1/5000 – Life Technologies - Grand Island-NY, USA), and 5 μl/ml penicillin/streptomycin/ml (Life Technologies).

Reverse transcriptase-polymerase chain reaction analysis for Bcl-2, Bcl-W, Fas, FasL, IL-12 and TGF-β

Total cellular RNA was isolated by modified protocol of acid guanidium thiocyanate/phenol/chloroform extraction [47, 48]. RNA was first reverse-transcribed into cDNA (Superscript^{tm} II – Gibco). PCR amplification was performed in a total volume of 50μl containing 2μl of the cDNA, 200ng of each primer, 500μM dNTP (Pharmacia Biotech – Piscataway-NJ, USA) and 1U Taq polymerase (Life Technologies) in buffer supplied by the polymerase manufacturer. Optimum PCR conditions were determined for each set of primers as follows: Bcl-2, Bcl-W, TGF-β, and cyclophilin, 40 cycles (58°C for 45s, 72°C for 90s, 95°C for 45s) Fas and FasL, 40 cycles (57°C 50s, 72°C for 90s, 95°C for 45s). Sample contamination by genomic DNA was verified by submitting the RNA sample to PCR amplification, omitting the RT step. The PCR products were submitted to 1.5% agarose gels electrophoresis containing ethidium bromide and visualized by excitation by ultraviolet light and digitally recorded using the Nucleovision® system (NucleoTech, San Mateo, CA, USA). The molecular weights of the products and the band pixel area were calculated using the Gel Expert® Software (NucleoTech) The semiquantitative expression (SE) of proteins was calculated for each sample using the following formula and expressed as arbitrary units (AU): SE = pixel area of the product to be analyzed/pixel area of cyclophilin x 100.

Statistical analysis

Comparisons of results were analyzed using the Kruskal-Wallis test (non-parametric ANOVA). Values of $p \leq 0.05$ were considered as indicative of significance.

Acknowledgements

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FIGURE 1

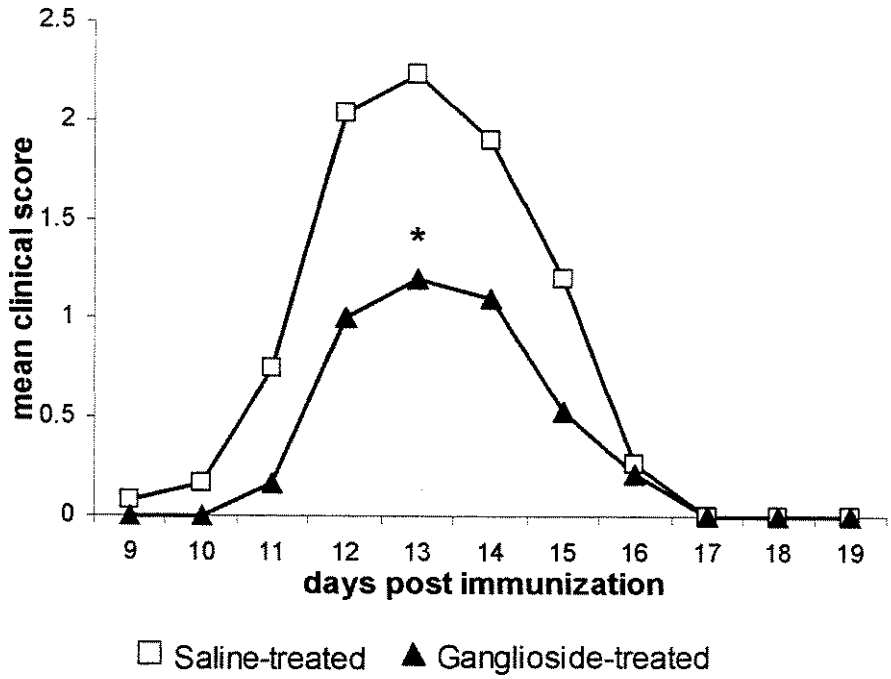

Figure 1. Ganglioside treatment suppresses clinical course of EAE. Animals inoculated with GP-MBP develop the signals of the disease at 9thdpi and demonstrate recovery after the 17thdpi. The ganglioside-treated group presented significantly milder neurological deficits($p= 0.0067$). In addition, the onset of clinical signs in this group was delayed. The data represent the mean clinical scores from all animals in each group. *, $p \leq 0.05$ *in vivo* treatments compared by the Kruskal-Wallis test (nonparametric ANOVA).

FIGURE 2

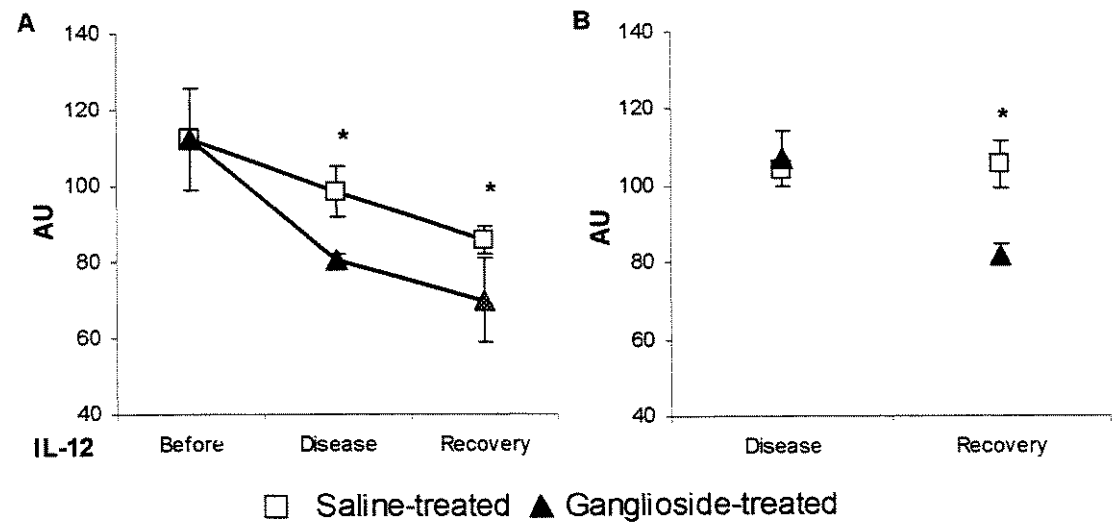

Figure 2. IL-12 mRNA expression in spinal cord and spleen cells from Lewis rats inoculated with GP-MBP. A) IL-12 mRNA expression in spinal cord demonstrates that IL-12 is involved with EAE genesis, since animals sacrificed at 7th dpi present up-regulated cytokine expression, which decreased during clinical signs and recovery. During the neurological signs, the animals treated with gangliosides present significantly lower levels (p= 0.01) of this cytokine. **B)** The analyses of IL-12 mRNA expression in spleen cells from animals with EAE demonstrate similar levels of this cytokine during clinical signs and a decrease after recovery. The animals treated with gangliosides present significantly lower IL-12 levels (p=0.05). **AU**=arbitrary units, mRNA levels as normalized by cyclophilin mRNA level (mRNA level/cyclophilin mRNA level), as quantified by image analyzer. Values are means ± SEM of three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$ *in vivo* treatments compared by the Kruskal-Wallis test (nonparametric ANOVA).

FIGURE 3

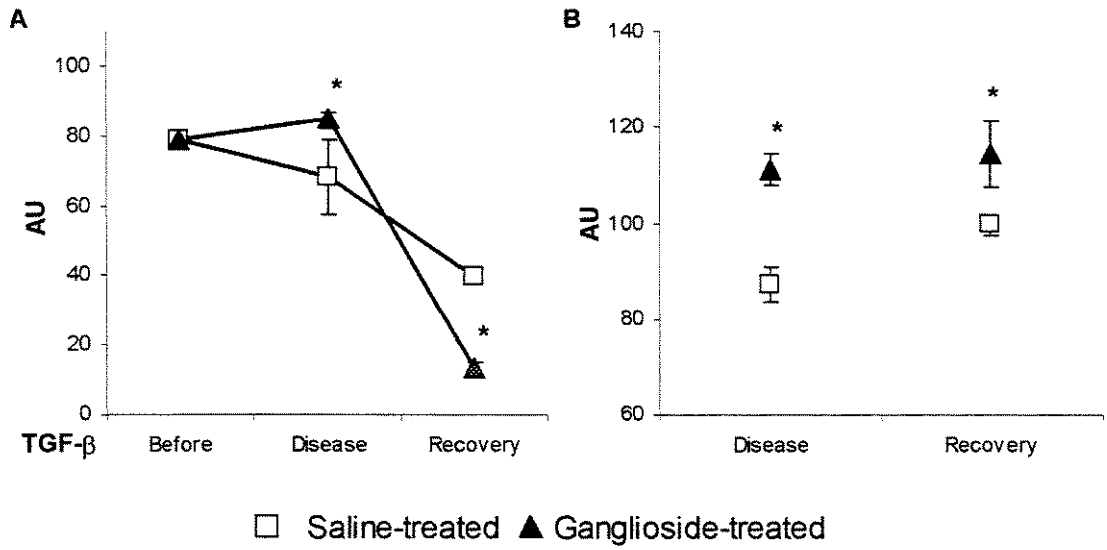

Figure 3. TGF-β gene expression. (Scales vary to improve observation of differences). **A)** In the spinal cord, the control group displays a decrease in levels of this cytokine, whilst the ganglioside-treated group present significantly (p=0.0085) higher levels of TGF-β. After recovery, both groups exhibit a decrease in these levels, and the animals treated with gangliosides show significantly lower levels (p=0.0001). Conversely, in spleen cells **(B),** the levels of this cytokine in the ganglioside-treated group are significantly higher both during clinical signs (p=0.02) and after recovery (p=0.03). **AU**=arbitrary units, mRNA levels as normalized by cyclophilin mRNA level (mRNA level/cyclophilin mRNA level), as quantified by image analyzer. Values are means ± SEM of three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$ *in vivo* treatments compared by the Kruskal-Wallis test (nonparametric ANOVA).

FIGURE 4

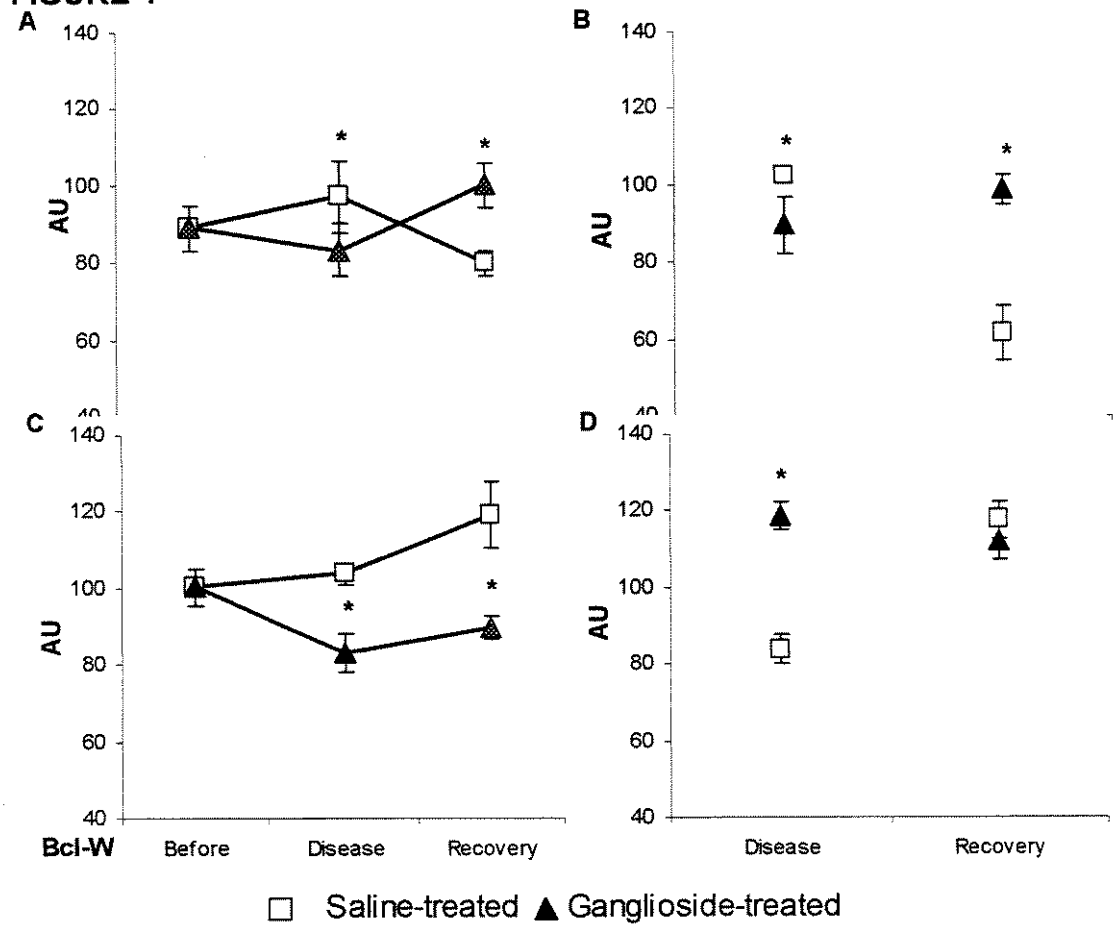

Figure 4. Gene expression of anti-apoptotic Bcl-2 family proteins. The survival factors are significantly reduced during clinical signs in spinal cord (A and C); Bcl-W is particularly reduced and its expression remains reduced after recovery. In spleen cells (B and D), the results demonstrate unchangeable Bcl-2 and Bcl-W levels during EAE in the ganglioside-treated group. In contrast, saline-treated exhibits a decrease of Bcl-2 levels after recovery, and an increase of Bcl-W after recovery. **AU**=arbitrary units, mRNA levels as normalized by cyclophilin mRNA level (mRNA level/cyclophilin mRNA level), as quantified by image analyzer. Values are means ± SEM of three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$ *in vivo* treatments compared by the Kruskal-Wallis test (nonparametric ANOVA).

FIGURE 5

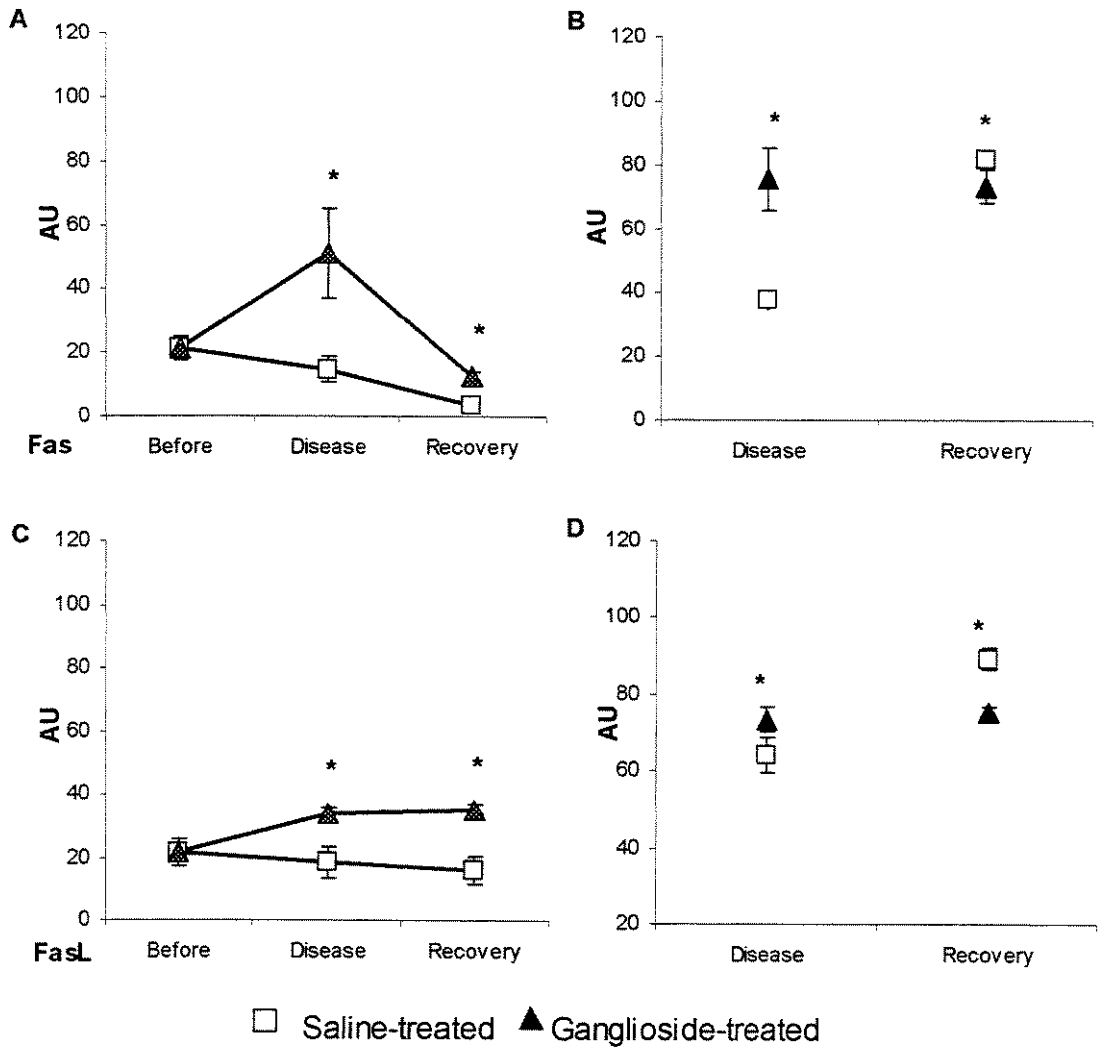
A
B
C
D
AU
120
100
80
60
40
20
0
Fas
FasL
Before
Disease
Recovery
*
Saline-treated
Ganglioside-treated

Figure 5. Gene expression of Fas and FasL. On the contrary to the results observed with survivor factors, the CNS expression of Fas and Fas-L (A and C) are up-regulated during EAE in the ganglioside-treated group and levels are significantly higher than for the saline treated group. In the spleen cells, similar results can be observed in animals treated with gangliosides, which have significantly higher levels of Fas and FasL during clinical signs than the control group. However, the Fas/FasL mRNA expression of the control group is significantly increased after recovery, whilst the levels of gangliosides are the same, suggesting that the modulation by gangliosides acts before the clinical signs peak. **AU**=arbitrary units, mRNA levels as normalized by cyclophilin mRNA level (mRNA level/cyclophilin mRNA level), as quantified by image analyzer. Values are means $\pm$ SEM of three separate experiments using spleen cells from three different donors. *, $p \leq 0.05$ *in vivo* treatments compared by the Kruskal-Wallis test (nonparametric ANOVA).

Figure 6

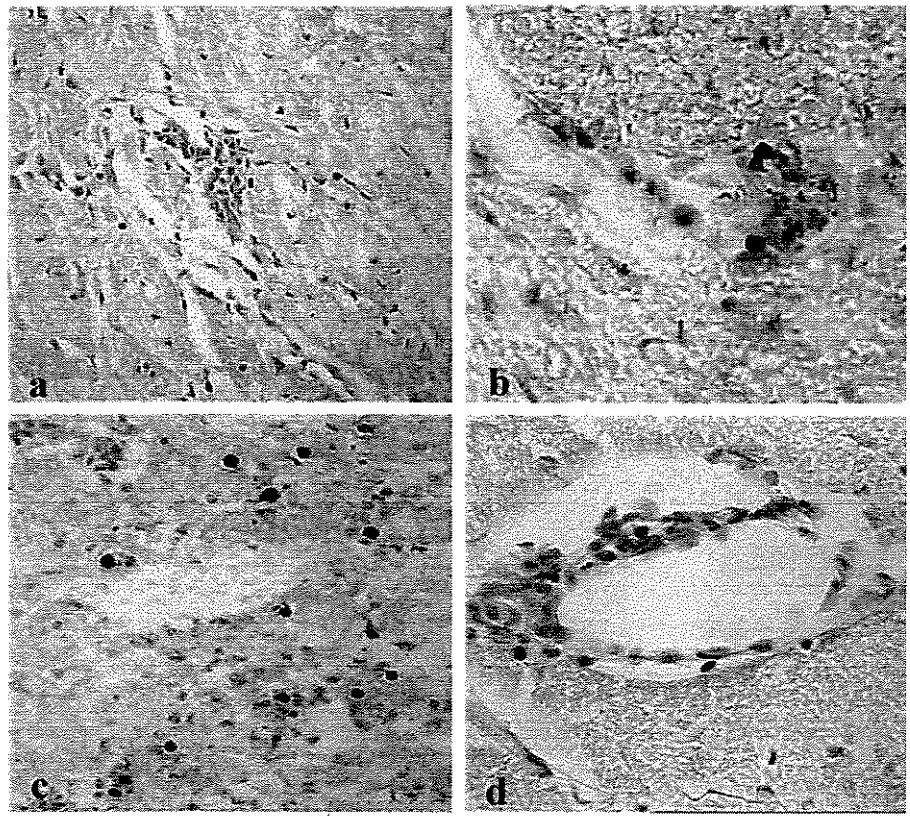

Figure 6. TUNEL analysis of *in situ* apoptosis in spinal cords of ganglioside and saline treated Lewis rats, immunized with GP-MBP. a) and b) sections from control group during clinical signs show few TUNEL$^+$ cells. The sections from animals treated with gangliosides exhibit considerable TUNEL$^+$ cells (c and d) particularly near to the blood vessel, in perivascular cuffs. Magnification a=20x, b, c, and d=40x.

DISCUSSÃO

O objetivo do presente trabalho foi avaliar tanto a ação imunomodulatória de gangliosídeos sobre a evolução clínica da EAE, como a expressão de citocinas moduladoras da doença e moléculas relacionadas com apoptose.

Ratos Lewis com EAE aguda foram tratados com altas doses de gangliosídeos (100mg/Kg) do 7º dpi ao15º dpi. Foram estabelecidos dois grupos controle: animais tratados com salina pelo mesmo período, ou animais sem tratamento. Os animais foram sacrificados antes da manifestação dos sinais clínicos (7º dpi), durante os sinais clínicos (15º dpi) e após a recuperação (19º dpi).

Os resultados evidenciam a ação imunomodulatória dos gangliosídeos sobre a manifestação dos sinais clínicos da EAE, visto que os animais tratados com estes compostos apresentaram deficiências neurológicas significativamente mais brandas que os grupos controle, sendo que alguns animais tratados com gangliosídeos não exibiram sinais clínicos da EAE. Além disso, a doença nestes animais apresenta curso reduzido, com início um dia após os animais dos grupos controle (10º dpi).

O efeito imunossupressor dos gangliosídeos tem sido demonstrado em vários modelos: na EAE aguda, na EAE crônica em camundongos NOD e na Encefalite Murina de Theiler, sugerindo que a administração diária destes compostos suprime significativamente as manifestações neurológicas da doença, bem como as lesões no SNC de forma dose-dependente (SHIMADA et al., 1994; INOUE et al., 1998; SEKIGUCHI et al., 2001)

Os resultados obtidos na avaliação clínica são corroborados pelo estudo *in vitro*. Em células esplênicas de animais tratados com gangliosídeos e sacrificados durante os sinais clínicos, observou-se significativa supressão na expressão de

citocinas Th1: IL-2, IFN-γ e TNF-α. Por outro lado, as citocinas Th2/Th3, relacionadas com a recuperação ou modulação da resposta imunológica, como IL-10 e TGF-β, estão elevadas em relação aos níveis encontrados no grupo controle. Células esplênicas de animais sacrificados após a recuperação da EAE, exibem redução na expressão de todas as citocinas.

As diferenças encontradas na expressão de citocinas reforçam a hipótese de que as propriedades imunomodulatórias dos gangliosídeos são tempo e dose-dependentes. Os resultados sugerem certa especificidade da ação dos gangliosídeos sobre linfócitos T auto-reativos, visto que após a recuperação, os seus efeitos são reduzidos, provavelmente pela diminuição da população linfocítica. Além disso, a ação destes compostos se dá em nível transcricional, com inibição específica de citocinas Th1 (IL-2, IFN-γ e TNF-α), sem alteração da expressão de citocinas Th2 (IL-4 e IL-10) (IRANI et al., 1996; KANDA, 1999; BUGGINS et al., 2001; SHEN and LADISCH, 2002; BHARTI and SINGH, 2003).

Os resultados obtidos da expressão de IL-12 e TGF-β *in* vivo, sugerem que a influência do tratamento de gangliosídeos sobre o padrão de expressão de citocinas encontrada em células esplênicas, reflita-se no SNC. Na medula espinhal de animais tratados com gangliosídeos, a expressão gênica da IL-12 está reduzida em oposição a expressão do TGF-β, que está elevada durante os sinais clínicos. Ou seja, houve supressão de citocinas que promovem o ataque imunológico contra o sistema nervoso central e aumento da expressão de citocinas regulatórias.

Os efeitos imunomodulatórios de gangliosídeos há muito são conhecidos. Acredita-se que estes compostos sejam responsáveis pela imunossupressão

observada em tumores, atuando como uma barreira protetora para a célula tumoral, ligando-se aos linfócitos e evitando o desenvolvimento de resposta imunológica competente (McKALLIP et al., 1999).

Os gangliosídeos podem diminuir a expressão de citocinas Th1 através da supressão dos primeiros eventos na cascata de transdução de sinais, bloqueando a mobilização do Fator Nuclear-κB (NF-κB), através da inibição da fosforilação e degradação da I-κB, proteína regulatória que liga-se ao NF-κB, impedindo sua translocação para o núcleo, impossibilitando a transcrição do RNAm de citocinas e promovendo a interrupção do ciclo celular (ZIEGLER-HEITBROCK et al., 1992; IRANI, et al., 1996; KANDA, 1999; BUGGINS et al., 2001).

O NF-κB regula os genes envolvidos na resposta imunológica como IL-2/IL-2R, TNF-α, MHC e IFN-γ. Quando inativo, este fator é mantido no citoplasma ligado a uma proteína inibitória denominada IκB, que se liga à região homóloga Rel no NF-κB bloqueando o "sinal de localização nuclear", evitando sua entrada no núcleo (WHITESIDE et al., 1997).

Uma vez fosforilado, o IκB sofre rápida poliubiquitinação e é degradado. Após sua liberação, o NF-κB tem sua "sequência de localização nuclear" exposta, possibilitando sua translocação para o núcleo, onde irá ligar-se ao DNA dando início à transcrição gênica (NG et al., 2002).

Estudos realizados com gangliosídeos de origem tumoral têm demonstrado que a fosforilação e degradação do IκB ocorre em alguns casos, tal fato sugere que os gangliosídeos possam induzir a degradação da forma ativa do NF-κB no

citoplasma antes de sua translocação para o núcleo (NG et al., 2002).

O ensaio de viabilidade celular mostrou, que células esplênicas de animais sacrificados durante os sinais clínicos e mantidas em meio de cultura com acréscimo de gangliosídeos, apresentam redução significativa quando comparado com os demais tratamentos *in vitro* (GP-MBP e não estimulado), independente do grupo experimental, após 48 horas em cultura.

Após 72 horas, embora com redução na viabilidade, as culturas mantidas com gangliosídeos, não apresentam diferença significativa nos grupos controle (não tratado e salina). Por outro lado, o ensaio de viabilidade em células esplênicas obtidas de animais sacrificados após a recuperação, mostrou redução significativa da viabilidade apenas em células tratadas com gangliosídeos de animais tratados *in vivo* com estes compostos.

Estes resultados reforçam o caráter dose e tempo-dependente da ação dos gangliosídeos, demonstrando que estes compostos têm atividade bio-reversível (IKEDA et al., 1992). Após sua incorporação à membrana plasmática, gangliosídeos exógenos podem ser metabolizados na via endocítica, como parte de endossomos tardios e lisossomos antes de sua degradação final (MÖBIUS et al., 1999).

A ativação de linfócitos T $CD4^+$ pode ser alterada por gangliosídeos exógenos, os quais ligam-se à membrana plasmática destas células, provocando alterações na orientação molecular do CD4 (OFFNER et al., 1987; HOON et al., 1989). Estas alterações disparam sinais que levam à dissociação da proteína-quinase não-receptora $p56^{LCK}$ da molécula de CD4, fazendo com que esta seja internalizada e degradada (SAGGIORO et al., 1993).

Acredita-se que a interação entre o gangliosídeo e o domínio amino-terminal da molécula de CD4 desencadeie a ativação da proteína quinase C-δ (PKC-δ) e fosforilação de resíduos de serina no domínio intracelular da molécula de CD4 promovendo a dissociação da molécula de CD4 e da proteinaquinase $p56^{LCK}$ e, consequentemente sua internalização e degradação (GOUY et al., 1995; GAROFALO et al. 1998).

Por outro lado, Krifuks (1998) e colaboradores demonstraram haver diferenças na supressão da molécula de CD4 segundo o tipo de gangliosídeo, uma vez que o GM1 parece atuar controlando a atividade de fosfatases celulares como a tirosina fosfatase CD45, a qual é responsável pela ativação da $p56^{LCK}$. Os autores observaram que a CD45 pode associar-se ao CD4 na superfície do linfócito T.

Em linfócitos T não ativados a $p56^{LCK}$ tem distribuição citoplasmática, associando-se à membrana plasmática após ativação em microdomínios especializados denominados "rafts", os quais atuam como plataformas para associação dinâmica de moléculas de sinalização, otimizando a resposta à ativação (KASAHARA and SANAI, 2000; TUOSTO et al., 2001).

Outro mecanismo proposto para ação de gangliosídeos sobre a viabilidade e proliferação celular é seu efeito sobre o ciclo celular através da proteína retiboblastoma (pRB). A pRB atua no controle do ciclo celular, mantendo sequestradas proteínas regulatórias que favoreçam a proliferação celular. Uma vez fosforilada a pRB sofre alteração em sua estrutura liberando as proteínas regulatórias que atuam na transcrição gênica, como a *c-myc*, induzindo proliferação celular. Gangliosídeos podem bloquear a proliferação de linfócitos T, através da

desfosforilação da proteína pRB, acarretando a interrupção do ciclo celular. Contudo, se o mecanismo de ação dos gangliosídeos atue na ativação de fosfatases ou na inativação de quinases não está esclarecido (IRANI et al., 1996; IRANI, 1998).

A supressão de citocinas Th1 envolve, também, a supressão de fatores de sobrevivência como IL-2, IL-12, IFN-γ, concomitante com o aumento na expressão de citocinas Th2/Th3, causando o desequilíbrio dos sinais de vida e morte o que, aliado à redução na viabilidade celular, sugerem a ação dos gangliosídeos na eliminação de células encefalitogênicas pela indução da morte celular por apoptose.

Para confirmação desta hipótese, foi avaliada a expressão de moléculas relacionadas com a sobrevivência celular (Bcl-W e Bcl-2) e com a indução de apoptose (Fas e FasL) na medula espinhal e células esplênicas isoladas de animais inoculados com GP-MBP antes da ocorrência dos sinais clínicos (7ºdpi), durante os sinais clínicos (15º dpi) e após a recuperação da doença (19ºdpi).

Pôde-se observar durante a ocorrência dos sinais clínicos, que a expressão gênica de Bcl-2 e Bcl-W no SNC de animais tratados com gangliosídeos é significativamente reduzida quando comparada a expressão nos animais do grupo controle. Os animais tratados com gangliosídeos, durante os sinais clínicos e após a recuperação, não apresentam alteração nos níveis de mRNA de Bcl-2 e Bcl-W nas células esplênicas. Por outro lado, os animais do grupo controle apresentam cinética antagônica. Coerente com as observações realizadas nos níveis de Bcl-W e Bcl-2, durante os sinais clínicos e após a recuperação, a expressão gênica de Fas e FasL é significativamente maior na medula espinhal de animais tratados com gangliosídeos. Em células esplênicas, na avaliação feita durante os sinais clínicos, os animais

tratados com gangliosídeos exibem níveis significativamente maiores de Fas e FasL. A expressão de Fas e FasL em células esplênicas observada nos animais do grupo controle (salina), está significativamente aumentada na fase de recuperação, quando comparada aos animais do mesmo grupo durante os sinais clínicos.

A técnica do TUNEL demonstrou que os animais tratados com gangliosídeos têm número substancialmente mais alto de células apoptóticas do que os animais do grupo controle. Estas células foram encontradas principalmente próximas aos vasos sanguíneos, o que sugere sua origem inflamatória.

Linfócitos T encefalitogênicos são eliminados do SNC por apoptose durante a EAE aguda, principalmente na fase recuperação clínica, fornecendo evidências que a recuperação espontânea da doença e o desenvolvimento da tolerância sejam dependentes deste processo (PENDER et al., 1992; PENDER and RIST, 2001).

A susceptibilidade de linfócitos T a apoptose está relacionada ao seu estado de ativação. Como os dados da literatura sugerem, gangliosídeos podem bloquear a translocação do NF-κB para o núcleo, tornando os linfócitos T mais susceptíveis à apoptose (KOLENKO et al., 1999).

Além da modulação de citocinas, gangliosídeos podem atuar diretamente na indução de apoptose. Após sua incorporação à membrana plasmática de timócitos adultos e fetais, verifica-se grande aumento na fragmentação de DNA, processo que pode ser revertido pelo uso de bloqueadores de endonucleases (ZHOU et al., 1998).

Outro mecanismo proposto para o efeito destes compostos no programa de morte celular é a ação direta sobre mitocôndrias, interagindo com o complexo III da cadeia transportadora de elétrons, o que provocaria estresse oxidativo, induzindo a

transição de permeabilidade e, consequentemente, a liberação de fatores apoptogênicos como citocromo *c* e do fator indutor de apoptose (AIF) e ativação de caspases (RIPPO et al., 2000; GARCÍA-RUIZ et al., 2000). A transição de permeabilidade envolve a abertura de um poro na membrana mitocondrial interna promovendo a difusão de moléculas maiores que 1500 Da, perda do gradiente protônico, inativação da fosforilação oxidativa e efluxo de cálcio da mitocôndria para o citoplasma, intumescência e rompimento da membrana mitocondrial externa, liberação de fatores indutores da apoptose, que estimulam a fragmentação nuclear e do citocromo *c*, que ativa a cascata de caspases responsáveis pela etapa final da apoptose (MALISAN and TESTI, 2002).

A ação dos gangliosídeos sobre os eventos do ciclo celular é dependente do tipo celular. Estes compostos podem resgatar neurônios do gânglio da raiz dorsal em processo de apoptose induzida. A expressão "de novo" de gangliosídeos em neurônios de portadores de Mal de Alzheimer indicam potencial regenerativo. Há vários ensaios clínicos que avaliam o potencial terapêutico destes compostos na regeneração nervosa (ARIGA et al., 1998).

Desta forma, nossos resultados permitem sugerir que a ação orquestrada por gangliosídeos atue por vias redundantes. Inicialmente inibindo a expressão de IL-2, IL-12, TNF-α e IFN-γ, estas citocinas além de perpetuarem a resposta imunológica, também atuam como fatores de crescimento, desta forma, a síntese de moléculas relacionadas com a sobrevivência celular como os membros anti-apoptóticos da família Bcl, como Bcl-2 e Bcl-w, também fica comprometida.

Por outro lado, níveis elevados de citocinas antiinflamatórias como IL-10 e

TGF-β, inibem a evolução da resposta imunológica, induzindo a apoptose em linfócitos T, através do o desequilíbrio entre fatores promotores e inibidores de morte celular.

Os resultados obtidos no presente trabalho trazem subsídios adicionais para o entendimento da ação imunomodulatória de gangliosídeos, além de abrirem perspectivas novas para linhas de pesquisa referentes aos mecanismos modulatórios da inflamação e vias de sinalização.

CONCLUSÃO

Considerando os resultados obtidos nos procedimentos experimentais:

1. Gangliosídeos têm ação supressora sobre a evolução clínica da EAE, uma vez que estes animais apresentam deficiências neurológicas significativamente menores que os animais tratados com salina ou não tratados.
2. A avaliação das citocinas *in vitro*, permite-nos inferir que estes compostos atuam em nível transcricional, com redução significativa das citocinas promotoras da doença como o IFN-γ e o TNF-α, concomitante com o aumento das citocinas supressoras da EAE, a IL-10 e o TGF-β.
3. O estudo de citocinas relacionadas com sobrevivência e indução de apoptose no sistema nervoso central mostra um aumento na expressão do TGF-β, citocina com caráter supressor e diminuição paralela da IL-12, citocina relacionada com estímulo e perpetuação da resposta inflamatória.
4. A viabilidade reduzida em cultura de células esplênicas tratadas com gangliosídeos *in vivo* e *in vitro*, juntamente com o número aumentado de células apoptóticas em infiltrados inflamatórios no SNC, sugerem que a apoptose seja um importante mecanismo da supressão exercida pelos gangliosídeos.
5. Estes resultados são coerentes com a avaliação da expressão de moléculas relacionadas com apoptose no sistema nervoso central, onde há o aumento na expressão de Fas e FasL, proteínas relacionadas com a indução da morte celular, e a diminuição da expressão de Bcl-W e Bcl-2, proteínas relacionadas com a sobrevivência celular.

6. Em conjunto, nossos resultados sugerem que os gangliosídeos atuam em diferentes mecanismos suprimindo a resposta imunológica, alterando a expressão de citocinas e induzindo a apoptose de linfócitos T auto-reativos.

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