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EFEITO DE UMA FRAÇÃO DERIVADA DO VENENO DE *Crotalus durissus terrificus* NA EVOLUÇÃO CLÍNICA DA ENCEFALOMIELITE EXPERIMENTAL AUTO-IMUNE E NA ATIVAÇÃO DOS LINFÓCITOS T

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Lista de Abreviaturas

AA: Ácido Araquidônico

APCs: Células apresentadoras de antígenos

CA: Componente A

CB: Componente B

CD: Conjuntos de Diferenciação

Cdt : *Crotalus durissus terrificus*

Con-A: Concanavalina A

COX: Ciclooxigenase

E.A.E: Encefalomielite Experimental Auto-imune

E.M: Esclerose Múltipla

IFN-γ: Interferon γ

IL: Interleucina

MBP: Proteína Básica de Mielina

MHC: Complexo Principal de Histocompatibilidade

MOG: Proteína Oligodendroglial de Mielina

PGE_2: Prostaglandina E_2

PHA: fitohemaglutinina

PLA_2: Fosfolipase A_2

PLP: Proteína Proteolipídica

S.N.C.: Sistema Nervoso Central

TCR: Receptor de Célula T

TGF-β: Fator de Crescimento e Transformação β

TNF-α: Fator de Necrose Tumoral α

Dedico este trabalho à minha mãe e à minha irmã. Afinal, as pessoas mais importantes de minha vida me ofereceram o apoio fundamental para conclusão desta obra.

RESUMO

O veneno da cascavel sul americana *Crotalus durissus terrificus (Cdt)* é uma mistura de proteínas incluindo crotoxina (ctx), que representa o principal componente neurotóxico do veneno. A crotoxina é composta de duas subunidades, uma básica, fortemente tóxica, reconhecida como sendo uma fosfolipase A_2 (PLA_2) e um componente ácido, não tóxico, denominado crotapotina.

O componente crotapotina possui atividade anti-inflamatória *in vivo*, demonstrada através de inibição de edema de pata de camundongos, induzido por carragenina. No presente trabalho, são apresentadas evidências de que macrófagos cultivados em presença de crotapotina liberam níveis aumentados prostaglandina E_2 (PGE_2), sendo que esse mediador inibe, significativamente, a resposta proliferativa de linfócitos.

O presente estudo também sublinha a importância do modelo animal para explorar formas alternativas de tratamento. Assim, mostramos que o tratamento com a crotapotina, reduz de forma significativa a gravidade da Encefalomielite Experimental Auto-imune (E.A.E). A EAE é uma doença desmielinizante causada por linfócitos T h1 auto-reativos à mielina, e seus componentes, e serve como modelo experimental de estudo da Esclerose múltipla. Parte do efeito protetor da crotapotina é devido à supressão da resposta proliferativa de linfócitos T encefalitogênicos, gerados pela imunização com o peptídeo da MOG. A diminuição da resposta proliferativa dos linfócitos não pode ser explicada pela alteração das moléculas co-estimulatórias CD80 e CD86 expressas na superfície dos macrófagos.

SUMMARY

The venom of the South American rattlesnake *Crotalus durissus terrificus* (*Cdt*) is a mixture of many proteins including crotoxin (ctx), which represents the major neurotoxic component of the venom. The crotoxin molecule is composed of two subunits, a basic, weakly toxic phospholipase A_2 (PLA_2) and an acidic, non-toxic subunit, denominated crotapotin.

The crotapotin component is reported to possess in vivo anti-inflammatory activity, which is demonstrated through inhibition of carrageenin-induced rat paw edema. This study provide evidence that macrophages cultured in the presence of crotapotin induce the release of prostaglandin E_2 (PGE_2), which inhibit the lymphocyte proliferative response. Since the Experimental autoimmune encephalomyelitis (EAE) model presents many clinical and histological similarities with multiple sclerosis, this study also underlines the importance of the animal model for exploring alternative forms of treatment.

We present evidence that the crotapotin significantly reduced the severity of EAE. Part of this protective effect is due to the suppression of the proliferative response of the encephalitogenic MOG 35-55 peptide-specific-T lymphocytes. Moreover, we demonstrated that the treatment with crotapotin did not modify the expression of costimulatory molecules (CD80 and CD86) on macrophage surfaces.

Introdução

Venenos de serpentes possuem uma elevada diversidade de componentes, cujas propriedades fisiológicas e farmacológicas têm sido amplamente estudadas. A importância dessas investigações pode ser notada, pelo uso bem sucedido, na clínica médica, de alguns peptídeos ou proteínas derivados de peçonhas, com atividade na hipertensão arterial, como é o exemplo de uma fração do veneno da *Bothrops jararaca*.

No Brasil, o pioneirismo dos estudos com toxinas isoladas de venenos de animais, deve ser atribuído a Karl Slotta e Heinz Fraenkel-Conrat, que em 1938 purificaram e cristalizaram o principal componente tóxico do veneno da *Crotalus durissus terrificus* (*Cdt*), a crotoxina (SLOTTA *et al.,* 1938).

No contexto dessa nova abordagem bioquímica, assumiu destaque a contribuição de Vital Brazil, para o esclarecimento dos efeitos farmacológicos da crotoxina, resultando em produção de relevantes trabalhos científicos, apresentados no Simpósio Internacional de 1966, no Instituto Butantan. Nessas obras, ele descreveu o bloqueio da transmissão neuro-muscular causado pela crotoxina, tendo observado uma depressão da resposta de diafragma de rato à acetilcolina (VITAL BRAZIL, 1966). Essa interferência pós sináptica, foi posteriormente explicada pela diminuição da sensibilidade à ação despolarizante desse neurotransmissor (BON *et al*., 1979). Coube também a Vital Brazil, a descrição dos efeitos pré-sinápticos da crotoxina (VITAL BRAZIL e EXCELL, 1971).

Além da atividade neurotóxica, a administração de crotoxina causava uma hemólise importante, dependendo da dose do veneno utilizada. Essa observação sugeria que essa proteína era composta por subunidades (HENDON *et a*l., 1970).

A hipótese de que componentes combinados formariam a principal toxina do veneno da *Cdt* foi confirmada através do uso de duas etapas cromatográficas, que permitiram o isolamento dos dois componentes do complexo crotoxina (Hendon *et al*., 1971). Um componente básico (CB) de 15000 daltons, ponto isoelétrico (p.i.) igual a 8,6 e reconhecido como sendo uma fosfolipase A_2 (PLA_2); um outro componente ácido (CA), possuindo um peso molecular de 9000 daltons, p.i. entre 3,4 e 3,7 e que foi chamado crotapotina. (BREITHAUPT *et al*., 1974).

O componente B detinha toda a atividade hemolítica, mas nenhum dos componentes mostrava o efeito neurotóxico. O problema foi desvendado pela observação de que a neurotoxicidade do complexo era recuperada após re-associação dos dois componentes (HENDON *et al*., 1971).

A crotapotina, embora aparentemente destituída de atividade biológica, comporta-se como uma proteína que impede a ligação do componente B aos sítios de baixa afinidade, favorecendo assim, que este alcance o seu alvo, a junção neuromuscular. Hoje, é conhecida a relação entre a alta toxicidade de venenos crotálicos e a presença de fosfolipase A_2 nos mesmos. No entanto, apesar da capacidade do componente A em potencializar a toxicidade do componente B do veneno da *Cdt*, quando dissociada do complexo crotoxina, a crotapotina inibe a atividade enzimática da PLA_2 (LANDUCCI *et al.,* 2000).

Se muitos esforços já foram dispensados para elucidação do potencial farmacológico de proteínas provenientes do veneno da *Cdt*, o mesmo não pode ser afirmado para o papel desses derivados sobre a resposta imunológica. Contudo, essa área é bastante atraente considerando a integração entre os sistemas biológicos e a possibilidade da descoberta de agentes terapêuticos.

Desde o início do século passado, há propostas referentes à utilização de venenos ofídicos como agentes anti-tumorais. Estudos, recentemente realizados, mostraram que a administração do veneno total da *Bothrops jarara* reduzia o crescimento de um tumor ascítico, conhecido como tumor de Ehrlich, induzido em murinos, possivelmente através da inibição na síntese de IL-6; no mesmo modelo de tumor, foi observado aumento na sobrevida dos camundongos, após o tratamento com o veneno total de serpentes de outro gênero (SILVA *et al*., 2002, ABU-SINNA *et al*., 2003).

Os resultados positivos, surgidos dessas tentativas, confirmam o potencial terapêutico do conteúdo total, ou de alguns componentes derivados de peçonhas, mas os mecanismos imunológicos relacionados a esses fenômenos ainda não são bem compreendidos.

Considerando–se a complexidade das interações dentro do sistema imune, os mecanismos de defesa contra os antígenos são didaticamente divididos em dois grupos principais: os mecanismos da imunidade inata e os da imunidade adaptativa.

Entende-se por *imunidade inata* o conjunto de elementos pré-existentes no organismo que lhe permite lidar com os antígenos internos e externos, e que não depende de reconhecimento baseado em moléculas que contam com regiões variáveis através das quais o antígeno é reconhecido. Nesse contexto, as células mais importantes são as células matadoras naturais (NK), os macrófagos e os granulócitos (neutrófilos, eosinófilos e os mastócitos), fazendo parte ainda, as proteínas do sistema complemento.

A *imunidade adaptativa* compreende o conjunto de elementos que permite uma imunidade específica, que se expande após a exposição a um estímulo antigênico e que se baseia na existência de células que portam moléculas com regiões variáveis, pelas quais o

antígeno é reconhecido. Nesse contexto, os linfócitos B (portam os receptores para o antígeno BCR) e T (portam os receptores para o antígeno TCR) representam um papel fundamental, bem como as células apresentadoras de antígenos (APCs: monócitos/macrófagos, as células dendríticas e os linfócitos B).

Durante o desenvolvimento do linfócito T no timo, ocorre a expressão de moléculas de superfície importantes para o fenômeno de reconhecimento do antígeno. São elas, as moléculas CD4 e CD8. No início do processo de maturação, os marcadores CD4 e CD8 são expressos no mesmo timócito. No final de sua maturação, a maioria dessas células só expressa um dos dois marcadores de superfície.

Os linfócitos T são capazes de interagir com o antígeno apenas se o mesmo for apresentado a ele como um peptídeo derivado do antígeno nativo, associado às moléculas do (MHC), na superfície de uma célula apresentadora de antígeno. A natureza do antígeno é fundamental nesse processo de apresentação. Antígenos endógenos, ou seja, normalmente presentes no citosol da célula apresentadora, são reconhecidos pelos linfócitos citotóxicos T CD8, juntamente com as moléculas MHC de classe I. Os antígenos exógenos, que normalmente são processados no interior de vesículas citoplasmáticas, são reconhecidos pelos linfócitos T CD4, quando apresentados por moléculas de MHC de classe II.

Em 1986, dois tipos de células T CD4 (Th1 e Th2) foram descritos, com base no padrão de citocinas secretadas por células representantes do sub tipo Th1 e pelos representados do subtipo Th2. Ensaios realizados com linhagens celulares, que são dependentes da presença de citocinas especificas para proliferarem, mostraram que as células Th1 secretam citocinas com atividade pro-inflamatórias como IFN γ, linfotoxinas, IL2, enquanto as células Th2 produzem citocinas com ação antiinflamatórias como IL 4, IL

5, e IL 10. Citocinas produzidas por um subtipo celular podem ter ação antagonista sobre as produzidas por outro subtipo (MOSMANN *et al*., l986).

As citocinas produzidas pelos linfócitos Th1, como o IFN γ, ativam macrófagos, coordenando a resposta imune contra antígenos intracelulares e, portanto, em conjunto com as células T citotóxicas compreendem os agentes da resposta imune celular. Por outro lado, as células Th2 participam da síntese e liberação de anticorpos pelas células B. Desse modo, a decisão sobre qual subtipo de células TCD4 prevalecerá, tem implicações profundas sobre os mecanismos imunes que serão desencadeados em resposta ao antígeno.

Basicamente, a ativação dos linfócitos T necessita de dois sinais. O primeiro, como já foi mencionado, é dado pela interação entre o peptídeo antigênico com as moléculas de MHC das células apresentadoras do antígeno, e o receptor para o antígeno presente nos linfócitos (TCR). O segundo sinal é dado pela ligação de moléculas co-estimulatórias presentes tanto nos linfócitos, como nas células apresentadoras do antígeno. No final da década de 80, foi demonstrado, pela primeira vez, que a ligação to TCR com o peptídeo no contexto da molécula de MHC, não era suficiente para a ativação dos linfócitos T. Esses autores observaram que os clones de linfócitos T que recebiam somente o sinal do TCR não se tornavam ativados, porém entravam em um estado de não resposta específica para o antígeno. Um segundo sinal, portanto, dado por moléculas co-estimulatórias é necessário para ativação dos linfócitos T (MUELLER *et al*., 1989).

As moléculas mais estudadas são CD28 e CTLA-4 (CD152) expressas nos linfócitos, que se ligam às moléculas CD80 e CD86 presentes nas células apresentadoras do antígeno. Há diferença quantitativa quanto à expressão dessas moléculas, nas diferentes células apresentadoras do antígeno. Células B e células dendríticas ativadas apresentam

maior densidade de moléculas CD86 do que moléculas CD80 em sua superfície celular (LENSCHOW *et al*., 1993; HATHCOCK *et al*., 1994; INABA *et al*., 1994).

O bloqueio da ligação das moléculas CD28/B7 inibe a resposta dos linfócitos T, mecanismo esse conhecido como anergia. A função ativadora da molécula CD28/B7 resulta da indução da transcrição da IL 2, na expressão do receptor para IL 2 (CD25) e a entrada da célula no processo de proliferação. A presença dessas duas moléculas nas células apresentadoras de antígeno CD80 e CD86, ambas se ligando à molécula CD28, estimulou estudos sobre a participação individual de cada uma delas, sugerindo que essas moléculas ativem de forma diferente, os linfócitos T. Utilizando anticorpos monoclonais contra as moléculas CD80 e CD86, foi proposto que a costimulação mediada por CD86 induzia a produção de IL 4 em células T *naive,* polarizando uma resposta do tipo Th 2 , enquanto a ativação via CD80 (B7.1) resultava na produção de IL 2 (FREEMAN *et al*., 1995). No entanto, estudos utilizando animais geneticamente modificados para essas moléculas, não mostraram diferenças na produção de citocinas (LEVINE et al., 1995; LANIER *et al*., 1995).

Já a ligação das moléculas CD80 e CD86 às moléculas CTLA (CD152) geram um sinal negativo na ativação de linfócitos.Uma das mais importantes provas da função inibidora da molécula CTLA4 veio de experimentos realizados em camundongos deficientes geneticamente para esta molécula, tendo esses animais desenvolvido uma desordem linfoproliferativa fatal (TIVOL *et al*., 1995). Essas observações mostraram que a molécula CTLA-4 regula negativamente e de forma crucial, a ativação linfocitária. Além disso, essa molécula é expressa em baixos níveis nas células não ativadas, tendo sua produção normalmente aumentada durante a ativação celular (LINSLEY *et* al., 1992,

WALUNAS *et al*., 1994); embora haja controvérsias, acredita-se que essa molécula regule negativamente a ativação linfocitária, através da estimulação da produção de citocinas com efeito anti-inflamatório como a IL 10 e o TGFβ (GOMES *et al*., 2000).

O curso dos processos da resposta imune adaptativa, portanto, é precocemente determinado por produtos de células pertencentes ao repertório que atua na resposta imune inata. As células TCD4+ oriundas do timo (células T *naive* ou Th0) podem, potencialmente, se transformar em qualquer um dos tipos de células Th funcionais. Alguns estímulos, geralmente produzidos durante a evolução da resposta imune inata, são capazes de induzir as células TCD4+ naive a se transformarem em Th1 ou Th2. Assim, a IL-12, presente no microambiente no qual a resposta imune está sendo elaborada, leva à expressão de determinados fatores de transcrição, que são responsáveis pela diferenciação das células TCD4+ em células Th1. Por sua vez, a IL-4 leva à expressão dos fatores de transcrição, que induzem a diferenciação de células TCD4+ em Th2 (SEDER e PAUL, 1994).

Assim, na dependência dos estímulos e do microambiente, os macrófagos podem controlar negativamente a resposta proliferativa de linfócitos através da produção de citocinas com efeito anti-inflamatório como a IL10, (FIORENTINO *et al*., 1991; IGETSEME *et al*., 2000; MOORE *et al*., 2001) e o TGFβ (ROBERTS *et al*., 1988) ou através da liberação de produtos do metabolismo do ácido araquidônico, como as prostaglandinas (SANTOS *et al.,* 1985; MUSATTI *et al*., 1987).

As prostaglandinas são metabólitos do ácido araquidônico e possuem importante papel no processo inflamatório. A fosfolipase A_2 cliva fosfolipídeos de membrana dando origem ao ácido araquidônico, que pode ser convertido em prostaglandinas e tromboxanos através da atividade enzimática da ciclooxigenase-1 (COX-1) ou pela ação da

ciclooxigenase-2 (COX-2). A COX-1 é constitutivamente presente em vários tipos celulares, enquanto a COX-2 é normalmente ausente em condições basais, podendo ser induzida por estímulos dados por LPS ou citocinas (NEEDLEMAN *et al*., 1986; SEIBERT *et al*., 1994).

Nas fases iniciais da inflamação, leucócitos são recrutados e migram através do endotélio vascular ao local-alvo. A passagem trans-endotelial das células brancas é possível, pela ação de citocinas capazes de alterar as propriedades adesivas do endotélio e pela ação da prostaglandina $E_{2,}$ sobre a permeabilidade vascular, dentre outros mediadores. Algumas citocinas envolvidas na inflamação, também estão sob o controle das prostaglandinas da série E_2 (PGE_2), o que explica em parte, a capacidade deste eicosanóide em modificar a resposta proliferativa de linfócitos (ECKMANN *et al*., 1997; LANENBACH *et al*., 1995; YAMADA *et al*., 1993; SEEERGEVA *et al*., 1997). As prostaglandinas atuam como potente indutor da função das APCs e na regulação da resposta dos diferentes subtipos de linfócitos T através de diminuição na produção de citocinas tais como a IL-12 e IFN-y (VAN DER POUW KRAAN *et al*., 1995; HILKENS *et al*., 1996) e de aumento na produção de IL-10 (HARIZI *et al*., 2002).

Em algumas situações, nas quais a resposta imune dominante é a mediada por linfócitos Th2, são encontrados níveis elevados de $PGE_{2.,}$ como é o caso, de pacientes infectados com o vírus da imunodeficiência humana (FOLEY *et al*., 1992; MASTINO *et al*., 1993), ou em camundongos Balb/c, (KURODA E YAMASHITA, 2003).

Em oposição ao importante papel protetor da resposta imune a antígenos potencialmente patogênicos, as reações de hipersensibilidades podem causar significativo

dano tissular e ao indivíduo de uma forma geral, em resposta a a antígenos inócuos ou aos antígenos próprios.

A autoimunidade pode ser entendida como uma resposta fisiológica do organismo. Os indivíduos convivem com linfócitos e os anticorpos dirigidos contra as estruturas próprias. A doença auto-imune, como a Esclerose múltipla (EM), pode surgir como resultado da quebra dos mecanismos de tolerância, que o organismo exerce sobre os clones de linfócitos auto-reativos.

A E.M. é uma doença inflamatória crônica do sistema nervoso central (SNC) que se caracteriza por apresentar infiltração de células imunes, destruição da bainha de mielina com perda eventual de oligodendrócitos (WAKSMAN, 1985, STEINMAN, 1996). As manifestações clínicas são muito variáveis, não existindo nenhum sintoma ou sinal específico da doença. Os indivíduos acometidos são adultos jovens, entre 20 e 45 anos, sendo que a doença manifesta-se raramente antes dos 15 ou após os 50 anos de idade. Sua evolução é imprevisível e polimórfica, conhecendo-se formas evoluindo em surtos (com ou sem remissão) ou cronicamente progressivas (NAVIKAS & LINK, 1996). A etiologia dessa condição é desconhecida, mas admite-se que seja multifatorial e de natureza auto-imune, onde o fator ambiental, provavelmente de origem infecciosa e a susceptibilidade genética parecem ter um papel essencial na sua determinação (STEINMAN, 1996).

Muito do conhecimento adquirido sobre a participação da resposta imunológica na E.M., se deve ao estudo desenvolvido no modelo experimental, a encefalomielite experimental auto-imune (EAE). A EAE começou a ser estudada após a descoberta da vacina contra a raiva por Pasteur, em 1875. Essa vacina, contendo componentes do cérebro do animal onde o vírus era cultivado, induzia nos indivíduos tratados, uma encefalite pós

vacinal, muitas vezes fatal. Atualmente, a EAE é considerada modelo experimental para o estudo dos mecanismos imunopatológicos nas doenças inflamatórias desmielinizantes de natureza auto-imune.

A EAE pode ser induzida em animais geneticamente susceptíveis, pela inoculação com mielina e seus componentes como a proteína básica de mielina (MBP), proteolipoproteína (PLP), glicoproteína associada à mielina (MAG), glicoproteína de mielina do oligodendrócito (MOG) e peptídeos encefalitogênicos derivados desses antígenos, ou ser transferida para animais normais por clones de linfócitos sensibilizados a estes componentes (BEN-NUN e COHEN, 1982; HOLOSHITZ *et al.,* 1983).

Embora exista alguns estudos mostrando a importância dos linfócitos Th2 na indução da EAE (LAFAILLE *et al*., 1997), a maioria dos trabalhos indicam que a doença é causada por linfócitos do tipo Th1, sendo que abordagens que polarizem a resposta imune para um padrão Th2, normalmente reduzem a severidade da doença.

Com relação ao tratamento dessas doenças, a imunossupressão global é normalmente utilizada no sentido de reduzir os danos causados pelas reações auto-imunes. No entanto, esse tipo de tratamento suprime a imunidade do organismo, levando a sérios efeitos colaterais. Existe, portanto, a necessidade de se encontrar novas medidas terapêuticas, que sejam mais específicas e, conseqüentemente menos danosas aos indivíduos. Assim, medidas imunoterapêuticas não específicas para o antígeno, como é o caso da administração de citocinas, e específicas, ou seja, o tratamento com os neuro-antígenos, tem sido gradativamente introduzidas na clínica médica.

A administração do Interferon beta a pacientes com esclerose múltipla está sendo utilizada, com sucesso, nos últimos 12 anos. Trata-se do primeiro tratamento, que

modificou o curso clínico da EM, na forma surto e remissão, desde o aparecimento dos corticoesteróides, acontecido no início da década de 70. Basicamente, o sucesso desse tratamento se deve ao aumento da resposta mediada pelo tipo Th2, contrabalançando a resposta Th1, normalmente dominante na E.M. e EAE e responsável pelos sintomas clínicos (RUDICK *et al*., 1996).

Recentemente, foi demonstrado que a administração de IFNγ, reduziu de forma significativa a gravidade da EAE, acompanhado de redução da resposta proliferativa dos linfócitos encefalitogênicos, com concomitante aumento na expressão das moléculas de CTLA4 (HALLAL *et al,* 2003).

Com relação a imunoterapia específica para o antígeno uma das muitas abordagens utilizadas, com sucesso, é a indução de tolerância oral aos neuro-antígenos. A administração oral de mielina ou de seus componentes, como a proteína básica de mielina ou o PLP, tem sido uma forma efetiva de se reduzir à gravidade da EM e da EAE. Indivíduos oralmente tolerizados com os neuroantígenos apresentam reversão no quadro clínico da doença, devido à maior expressão de TGF-β, IL4, PGE_2 e IL-10 (WEINER *et al*., 1994; FARIAS *et al*., 2003).

A possibilidade de se utilizar frações de veneno com o objetivo de diminuir os danos das reações auto-imunes, parece um alternativa interessante, uma vez que a atividade antiinflamatória já foi previamente descrita.

Estudos realizados por Cardoso e Mota (1997) sugerem que a crotoxina e o veneno total da *Cdt* reduziam a resposta imune humoral. Os autores mostraram que a inoculação de camundongos com ovalbumina (OVA) ou albumina de soro humano (HSA)

induzia a produção de anticorpos IgG1, mas que a inoculação de crotoxina ou do veneno total da *Cdt* inibe a produção deste anticorpo (CARDOSO e MOTA, 1997).

As observações de Cardoso e Mota foram reforçadas em experimentos nos quais o efeito imunossupressor do veneno total de *Cdt* e do componente crotoxina foi confirmado e comprometido através de aquecimento a determinadas temperaturas (RANGEL *et al*., 2000). Trabalho ainda mais atual, procura explicar o papel da crotoxina sobre as reações imunes através da quantificação dos níveis de citocinas e de corticosterona. Animais tratados com crotoxina apresentaram aumento nos níveis de IL-10, IL-6 e corticosterona, quando comparado com animais não tratados (CARDOSO *et al.,* 2001).

Dessa forma, foi nosso objetivo estudar o efeito da fração crotapotina sobre a resposta proliferativa de linfócitos e sobre a evolução clínica de EAE.

Objetivos:

1- *Estudar o efeito da crotapotina sobre a ativação de linfócitos T:*

   1.1. Resposta proliferativa de linfócitos a estímulo mitogênico;
   1.2. Liberação de PGE_2 por macrófagos.

2- Estudar o efeito da crotapotina sobre a evolução clínica da Encefalomielite Experimental Auto-imune

   2.1. Avaliação da evolução clínica da E.A.E. em camundongos tratados ou não com crotapotina;
   2.2. Resposta proliferativa de linfócitos a estímulo antigênico;
   2.3. Expressão de moléculas co-estimulatórias.

Crotapotin, a snake venom protein, as therapeutic approach to prevent the Experimental autoimmune encephalomyelitis

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Key words: demyelination, T cell activation, costimulatory molecules

Running title: Crotapotin reduces the severity of EAE

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ABSTRACT

The antiinflammatory property has been demonstrated for the venom of the *Crotalus durissus terrificus (cdt)*. We previously demonstrated that one fraction of *Cdt*, crotapotin, inhibited the proliferative response of lymphocytes *in vitro* by inducing the secretion at high levels of PGE_2 by macrophages. Here we present evidence that the crotapotin also significantly reduced the severity of Experimental Autoimmune Encephalomyelitis. Part of this protective effect is due to the suppression of the proliferative response of the encephalitogenic MOG 35-55 peptide-specific-T lymphocytes. Our data also demonstrated that the treatment with crotapotin did not modify the expression of costimulatory molecules (CD80 and CD86) on macrophage surfaces, this suggest that effect of the crotapotin on the clinic evolution of the E.A.E. have not been associate the anergy to self-reactive clones of T cells.

INTRODUCTION

Experimental Autoimmune Encephalomyelitis (EAE) is a demyelinating disease of the central nervous system that serves as a model for multiple sclerosis (Ben-Nun & Cohen, 1982; Holoshitz et al., 1983). The adoptive transfer of encephalitogenic T lymphocytes has demonstrated that the EAE is caused by CD4 T lymphocytes. Two signals are needed for T cell activation. The binding of the T cell receptor to a peptide-MHC complex provides the first, while the second is provided by costimulatory proteins. These costimulatory molecules which help determine T cell responses (CD80 (B7.1) and CD86 (B7.2) are located on antigen-presenting cells, they bind to CD28 and CD152 (CTLA4) on T cells. The coupling of costimulatory molecules with the peptide signal through the T cell receptor (TCR) induces T cell proliferation and cytokine secretion. CTLA4 is a negative regulator of T cell activation.

Since the EAE model presents many clinical and histological similarities with multiple sclerosis, this study underlines the importance of the animal model for exploring alternative forms of treatment. An approach using snake venom as a possible therapeutic agent has been described for many pathologic conditions such as neoplasms, diabetes and experimental demyelinating disease such as EAE (Lewis and Garcia, 2003).

The venom of the South American rattlesnake *Crotalus durissus terrificus* (*Cdt*) is a mixture of many proteins including crotoxin (ctx), which represents the major neurotoxic component of the venom (Slotta et al., 1938). The crotoxin molecule is composed of two subunits, a basic, weakly toxic phospholipase A_2 (PLA_2) and an acidic, non-toxic subunit, denominated crotapotin (Hendon and Fraenkel-Conrat, 1971).

The crotapotin component is reported to possess in vivo anti-inflammatory activity, which is demonstrated through inhibition of carrageenin-induced rat paw edema (Landucci et al., 1995). Recently, it has been demonstrated that normal macrophages cultured in the presence of crotapotin induce the release of prostaglandin E_2 (PGE_2), which inhibit the lymphocyte proliferative response (Garcia et al., 2003).

Since the EAE is caused by T lymphocytes, the present study investigates whether crotapotin administration interferes in the clinical evolution of EAE and the activation of these T lymphocytes.

Materials and Methods

Animals: Six to eight-week old female C57BL/6 mice were obtained from The Harlan Sprague Dawley Laboratory. The animals were housed and maintained pathogen free in the University animal facility. All procedures were carried out in accordance with the guidelines proposed by the Brazilian Council on Animal Care (COBEA) and approved by the Ethical Committee on Animal Experimentation (CEEA/UNICAMP).

Treatment with crotapotin: The mice were immunized and then divided into three groups of 10; one received no treatment and one received ovalbumin (OVA) (controls), while the other received crotapotin of 3μg/mouse (9 times) via IP after immunization with MOG peptide/CFA.

Antibodies: Mouse Monoclonal antibodies anti- CD80 and CD86 mAb, conjugated to FITC, were purchased from PharMingen, San Diego, CA, USA. MOG peptide 35-55 (M E V G W Y R S P F S R V V H L Y R N G K) was purchased from Genemed Synthesis CA, USA.

Immunization and induction of EAE: All mice were immunized with MOG peptide. Each animal received an in flank injection of 60 μg MOG in 0.10 ml of PBS emulsified in an equal volume of complete Freund´s adjuvant (CFA) containing 4 mg/ml of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). The animals also all received 200 ng of pertussis toxin i.p. (List Biologic Laboratories, Campbell, CA, USA) The mice were then evaluated daily for signs of disease and graded on the following scale: grade 1, limp tail; grade 2, hind limb weakness; grade 3, plegia of both hind limbs; grade 4, plegia of three or four limbs; grade 5, moribund.

Isolation of leukocytes: Spleen and draining lymph node cells were minced through a steel sieve in Hank´s buffer. Connective tissue fragments were allowed to settle for 10 min at 4^0 C and lymphocytes were sedimented from the supernatant by low speed centrifugation (170 x g, 10 min, 4^o C).

Proliferation assay: For MOG peptide stimulation, lymphocytes were cultured in 1640 RPMI with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L - glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 2% heat-inactivated fetal bovine serum and $5xl0^{-5}$M 2-Mercaptoethanol (Sigma Chemical Co. St. Louis, MO, USA). The optimal concentration of the peptide, determined in pilot experiments, involving various concentrations, was 10 μg/ml. The lymphocytes ($2x10^5$/well) were cultured for 96 h; the cultures were pulsed with 1 μCi (^{3}H) thymidine (Amershan, Buckinghamshire, UK) per well during the last 16 h of culturing and then harvested (Cambridge Technology, Cell Harvester, Cambridge, MA, USA). Thymidine uptake was measured in a scintillation counter (Beckman System, San Jose, CA, USA).

Obtention of macrophages: Spleen cells from treated and control mice were adjusted to a concentration of $2x10^6$/ml and incubated for 2 h at 37^oC on plastic plates for adhesion. This procedure yielded 90% of purified macrophages (data not shown). The cells were then removed for staining with anti CD80 and CD86.

Flow cytometry. Single cell suspensions ($1x10^6$cells/ml) were stained using anti-CD80 and CD86 mAb, conjugated with FITC, as well as the irrelevant antibody isotype control. The analysis was performed using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA, USA).

Histological analysis: Spinal cords were collected at various time points after immunization with MOG peptide. For haematoxylin and eosin staining, the animals were deeply anaesthetised and transcardially perfused with heparinized 0.9% saline and 4% paraformaldehyde in 0.1M phosphate buffer (PB) pH 7.4. The spinal cord was dissected out and left overnight in the same fixative. The specimens were then processed for paraffin embedding and transversal 8 μm serial sections were mounted onto silane-coated slides.

Statistical analysis: The statistical significance of the results was determined using a analysis of variance (ANOVA), Kruskal Wallis test. A *p* value smaller than 0.05 was considered to be significant

RESULTS

Reduction of clinical signs of EAE by administration of crotapotin

The administration of crotapotin resulted in a delay in the onset of the disease and a much milder course for the disease (maximum clinical scores of 3.5 for OVA treated mice, 3.0± 0.80 for immunized animal and 0.2 ±0.4 for crotapotin treated mice (Figure 1). No significant difference in animal weight was noted between the crotapotin-treated and controls groups.

Cell infiltration of the central nervous system

Mice spinal cords were removed from the three groups during the acute phase of the disease and stained. The region cervical of the spinal cord showed infiltration of mononuclear cells in the control group (Figure 2A and 2C), whereas inflammatory infiltration was not observed in the spinal cord from mice treated with crotapotin (Figure 2B e 2D).

Inhibition of T cell proliferation by crotapotin

In vivo administration of crotapotin significantly reduced the proliferative response of lymphocytes stimulated with MOG 35-55 peptide (1 and 5 μg/ml respectively): immunized mice: 7800±840 and 7600±480 cpm; OVA treated control: 6400±1200 and 4800±940cpm ; CA treated mice 2400±1200 and 1900±1200 cpm. There are significant ($p<0.001$) reduction in the proliferative response in CA treated mice compared to controls (Figure3).

Modification of expression of costimulatory molecules by in vivo administration of crotapotin

Since crotapotin administration after MOG peptide immunization lessened the severity of EAE and suppressed the *in vitro* proliferative response, the ability of crotapotin to modify costimulatory molecule expression was examined. The *in vivo* administration of crotapotin did not modify the expression of CD80 in the treated group (33.07% in immunized group and 26.26% treated groups, respectively ($p>0.05$). Moreover, there was no significant modification observed in the expression of CD86 in the three groups ($p>0.05$, Figure 4).

DISCUSSION

This study has shown that the *in vivo* administration of crotapotin in mice reduces the severity of EAE. As demonstrated in Figure 1, the administration of this component markedly reduced the clinical signs of EAE when given after immunization, resulting in a stable, less severe form of the disease, whereas the control animals continued to have relapses of the acute form of the disease. The effectiveness of treatment was confirmed by a histological examination of the CNS, which revealed the absence of inflammatory mononuclear cell infiltration the treated mice (Figure 2B e 2D).

The *in vivo* administration of crotapotin alters the *in vitro* proliferative response of MOG peptide-specific T lymphocytes, as demonstrated in Figure 3. This proliferative response is one of the most useful methods for the evaluation of the functionality of T lymphocytes. Although the proliferative response and the subsequent differentiation of effectors T cells requires interactions between T lymphocytes and macrophages, this latter can also suppress this response. This suppressive effect may be attributed to increase in the production of anti-inflammatory cytokines such as TGF-β and IL10 (Fiorentino et al., 1991; Igetseme et al., 2000; Moore et al., 2001; Roberts et al., 1988) or to the production of inflammatory mediators such as Prostaglandin E_2 (PGE_2) (Van der Pouw Kraan et al., 1995; Hilkens et al., 1996). In normal macrophages this mediator significantly reduced the proliferation of normal T lymphocytes. In this paper, the administration of crotapotin, was shown to reduce the proliferative response of encephalitogenic T lymphocytes and its severity. This suppressive effect on T lymphocytes may be attributably to an increase in the

production of PGE_2 in the central nervous system. In fact, an increase in the production of PGE_2 by brain macrophages has been described during the recovery phase of the EAE induced in the Lewis rats (Khoury et al 1992).

Not only the participation of macrophage products in the proliferative response, but also the expression of costimulatory molecules CD80 and CD86 on macrophages after treatment with crotapotin was investigated. The interaction between CD28 on the T-lymphocyte and the CD80 (B7.1) or CD86 (B7.2) on the antigen-presenting cells generate the most important costimulatory signal. It has been suggested that CD80 and CD86 play a differential role in setting the balance between Th1 and Th2 responses, with CD80 activating the Th1 response and CD86 the Th2 response. In the EAE model, antibody to CD80 have been shown to inhibit Th1-mediated EAE in mice, whereas the presence of the antibody to CD86 made the clinical disease worse (Kuchroo et al., 1995). However, other data do not support this hypothesis, since mice deficient in either CD80 or CD86 were susceptible to EAE, although those deficient in both molecules were resistant to the disease (Chang et al. 1999). In the present study, we observed that treatment with crotapotin did not change the expression of CD80 and CD86 on macrophages from the spleen of treated mice, although there was a reduction in the severity of the disease, as well as a diminished proliferative response to the encephalitogenic peptides. Previous research suggests that the differential function of costimulatory molecules is mediated by their differential locations and the kinetics of their expression, rather than distinct unique function of individual molecules. Macrophages that migrate to the CNS express costimulatory molecules differently than peripheral macrophages. The mere infiltration of T cells alone into the CNS is not sufficient for the induction of EAE (Kojima et al., 1997), but it has been suggested

that such infiltrating T lymphocytes recruit peripheral macrophages, and these latter cells may to cause tissue damage.

It has been demonstrated that during the course of EAE, CD86 expression in the CNS is correlated with clinical signs, while CD80 is expressed only during remission. In the periphery, CD80 expression on antigen-presenting cells peakes with the clinical disease (Issazadeh et al., 1998). Since cells were analyzed during the acute phase of the disease, the data obtained here is in agreement. Costimulatory molecules regulate Th cytokines production and these cytokines may also regulate the expression of costimulatory molecules during the immune response. The results presented here suggest that an increase in anti-inflammatory cytokines and mediators such as PGE_2 in the localization *in vivo* of the inflammatory response is induced by treatment with crotapotin, altrough this expression varies depending on whether it occurs in the CNS or peripheral organs.

In conclusion, it has been demonstrated here that therapy with crotapotin markedly reduces the proliferative response of encephalitogenic T lymphocytes without causing modification in the expression of costimulatory molecules on peripheral macrophages, these alterations in T cell activation result in a significant reduction in the severity of EAE.

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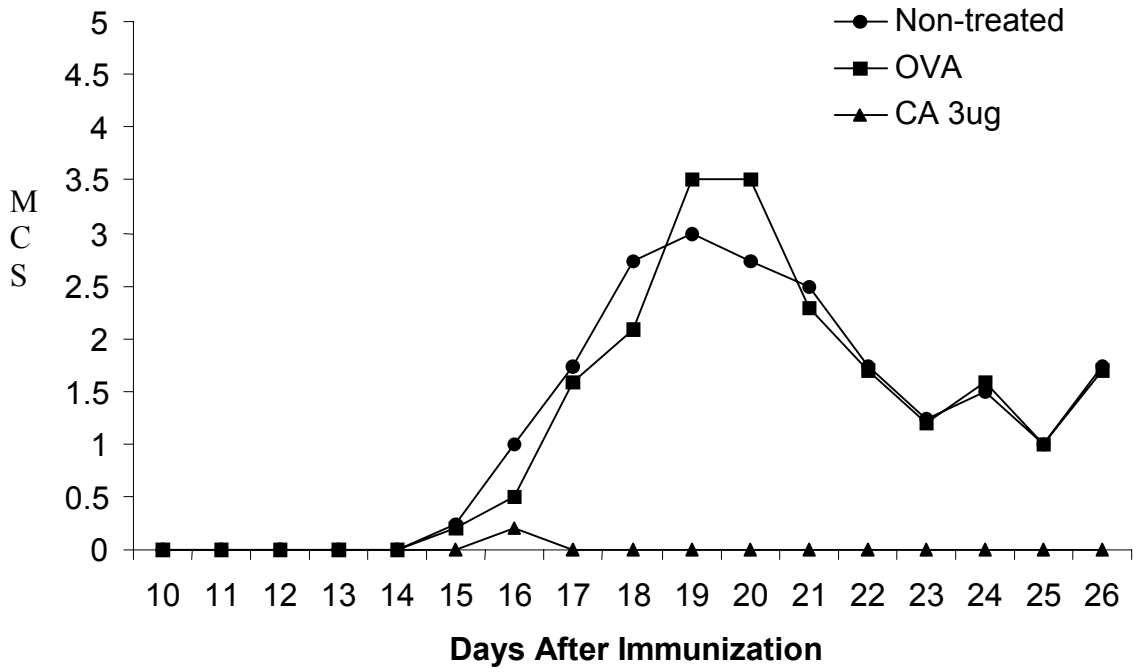

	Day of Maximal Mean Score	*Maximal Score*	*Maximal Mean Score*	*SD of Maximal Mean Score*
Non-treated	19	4	3	0.816497
OVA 3µg (9 doses)	19	3,5	3,5	0
CA 3µg (9 doses)	16	1	0,2	0.447214

Figure 1

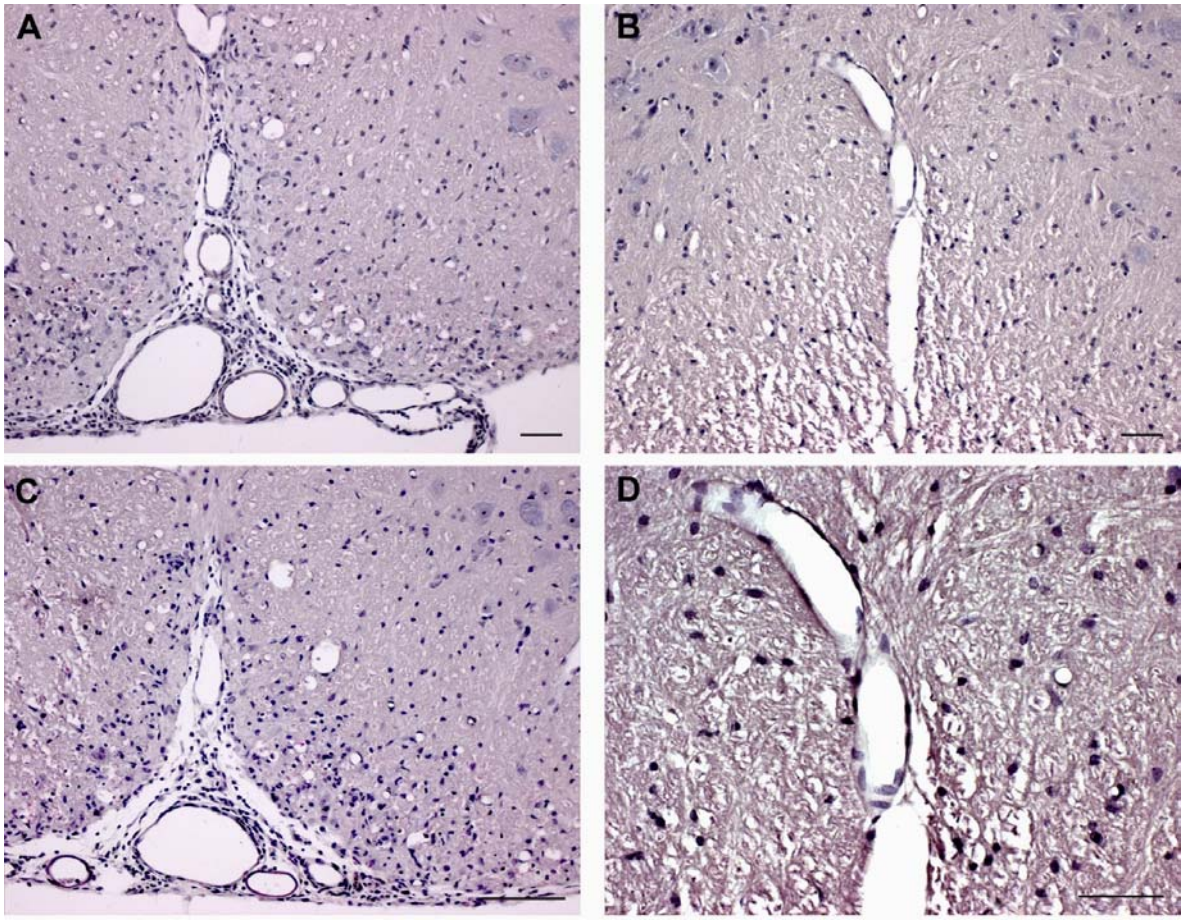

Figure 2

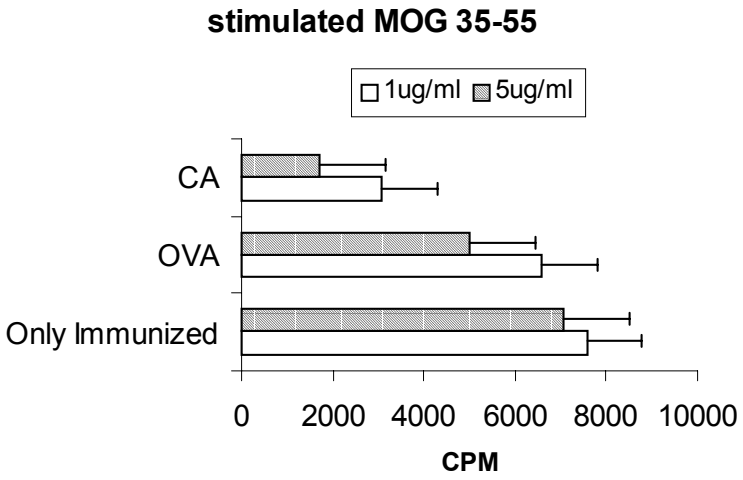
stimulated MOG 35-55
1ug/ml
5ug/ml
CA
OVA
Only Immunized
0
2000
4000
6000
8000
10000
CPM

Figure 3.

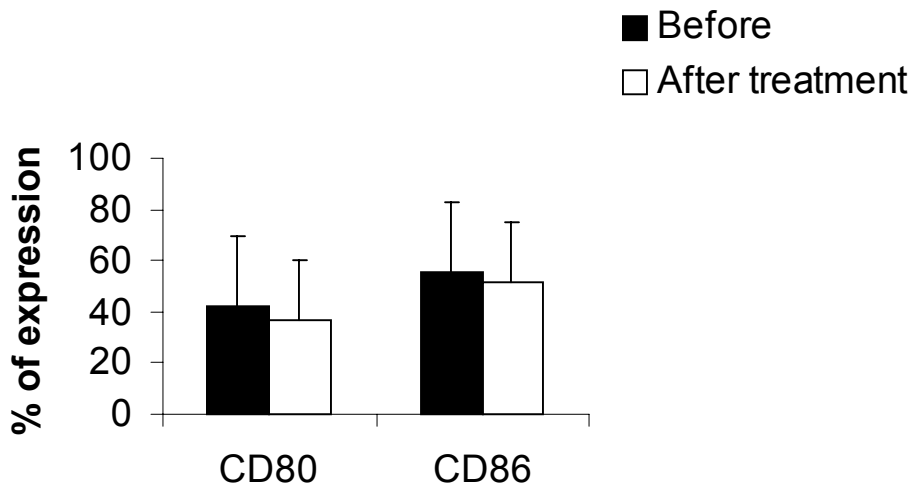
Before
After treatment
% of expression
100
80
60
40
20
0
CD80
CD86

Figure 4.

FIGURE CAPTIONS

Figure1. Mean Clinical Scores (MCS) of EAE in mice treated *in vivo* with crotapotin (CA) and OVA. The mice were divided in three groups; all were then immunized with MOG-peptide/CFA. The others two groups were treated with CA or OVA (3ug/mouse/9times).

Figure 2. Photomicrographs of HE staining of mouse spinal cords sections obtained during the acute phase of the disease. A: immunized group, B and D: immunized and crotapotin treated group, C: immunized and OVA treated group.

Figure 3. Effect of crotapotin on proliferative response of spleen cells stimulated with MOG 35-55 peptide. Draining lymph node cells from mice treated *in vivo* with crotapotin were stimulated *in vitro* with MOG peptide (1ug/ml and 5ug/ml).

Figure 4. Costimulatory molecule expression on macrophages of mice before and after treatment with crotapotin. Spleen cells from naïve mice, as well as those before and after crotapotin treatment were labeled with monoclonal antibodies to CD80 and CD86, and the expression of these molecules was quantified by flow cytometry.

CONCLUSÕES

- Crotapotina in vitro inibe a resposta proliferativa de linfócitos à Con-A através de indução de aumento na síntese de PGE_2 por macrófagos (Garcia et al., 2003);
- Crotapotina reduz a severidade da EAE através de inibição da resposta proliferativa de linfócitos encefalitogênicos, possivelmente por causar elevação nos níveis de PGE_2 nos órgãos linfóides periféricos e/ou no SNC;
- Crotapotina exerce efeito supressor sobre a resposta imune celular e na evolução clínica da EAE sem afetar a expressão de moléculas co-estimulatórias (CD80 e CD86) sobre macrófagos periféricos.

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Crotapotin induced modification of T lymphocyte proliferative response through interference with PGE_2 synthesis

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Abstract

The immunosuppressive property has been demonstrated for the venom of the *Crotalus durissus terrificus*. Using a simple, novel method for obtaining crotapotin and phospholipase A_2 isoforms from venom, it was possible to demonstrate that the addition of crotapotin to cultures of isolated lymphocytes resulted in a significant inhibition of the cellular proliferative response to Concanavalin A. This reduction in blastogenic response of lymphocytes is accompanied by a significant increase in the production of PGE_2 by macrophages. This effect on the innate immune response suggests that this compound may modify the subsequent adaptative immune response.

Keywords: T cell activation; Inflammatory response; Crotapotin

1. Introduction

The venom of the South American rattlesnake *Crotalus durissus terrificus* is a mixture of many proteins, including crotoxin, which is the major neurotoxic component of the venom. The crotoxin molecule is composed of two subunits, a basic, weakly toxic phospholipase A_2 (PLA_2) and an acidic, non-toxic subunit, known as crotapotin (Slotta and Fraenkel-Conrat, 1938).

Pharmacologically, the crotapotin component is thought to act as a chaperone protein for PLA_2 to increase the biological activities of this enzyme (Bon et al., 1979). Moreover, it has been reported to possess in vivo anti-inflammatory activity, as seen in the inhibition of carrageenin-induced rat paw edema (Landucci et al., 1995).

In the present study, the effects of this acidic fraction of the venom on the proliferative response of lymphocytes were investigated since this in vitro proliferative response is one of the most reliable tests for evaluating the functionality of T lymphocytes. This response, as well as the subsequent differentiation into effector T cells, involves interactions between T lymphocytes and macrophages, both of which have been shown to suppress various types of cell-mediated immune functions.

Given the intimate liaison between crotapotin and the PLA_2, as well as the role of the latter in the production of arachidonic acid (AA), an investigation was made of a possible link between the reported anti-inflammatory activity of this protein and the products of metabolism of AA.

AA is produced by the action of PLA_2 on the membrane phospholipids of most cells. Free AA can then be converted through distinct enzymatic pathways (COX 1 and COX 2) into the eicosanoids involved in the inflammatory response (Needleman et al., 1986). It is well established that the induction of the isoform COX 2 leads to the biosynthesis of E_2 prostaglandin (PGE_2) by activated monocytes/macrophages (Seibert et al., 1994; Musatti et al., 1987). PGE_2, which is a potent vasodilator (Cohn et al., 1997; Sheng et al.,

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1997), inhibits mitogen-induced lymphocyte responses, thus preventing overstimulation of the cellular immune response (Eckmann et al., 1997; Lanenbach et al., 1995; Yamada et al., 1993; Sergeeva et al., 1997). Macrophages/monocytes are considered to be the major source of PGE_2 since classical observations have demonstrated that normal human monocytes suppress both the phytohemagglutinin (PHA) and antigen-induced lymphocyte proliferative response when the monocyte–lymphocyte ratio increases (Passwell et al., 1982; Santos et al., 1985). Moreover, the addition of indomethacin, an inhibitor of prostaglandin synthesis, to cultures of mononuclear cells stimulated with PHA results in a significant increase in this proliferation (Musatti et al., 1987; Santos et al., 1985).

Since the prostaglandins synthesized as a result of the action of PLA_2 play such an important role in the control of the lymphocyte proliferative response, any protein which is intimately linked to this enzyme should be investigated in relation to possible effects on the cellular immunity system. The effects of crotapotin on this system are thus investigated in the present paper.

2. Materials and methods

Animals. Six- to eight-week-old female SJL mice were obtained from The Harlan Sprague Dawley Laboratory and housed and maintained pathogen free in the university animal facility. All experimental procedures were done according to the guidelines proposed by the Brazilian Council on Animal Care (COBEA) and approved by the Ethical Committee on Animal Experimentation (CEEA/UNICAMP).

Venom. C. durissus terrificus venom was furnished by the Instituto Butantan (São Paulo, Brazil).

Reverse phase high pressure liquid chromatography (RP-HPLC). Twenty milligrams of desiccated whole venom were dissolved in 750 μl of 0.1% (v/v) trifluoroacetic acid (Solvent A). The resulting solution was clarified by centrifugation at 10,000 rpm for 3 min and the supernatant applied to a μ-Bondapack C18 column ($78 \times 30\ cm^2$) (Waters 991-PDA system). The column was eluted with a non-linear gradient (0–66.5%, v/v) of acetonitrile (Solvent B) at a flow rate of 2.0 ml/min. Absorbances were monitored at 280 nm and the fractions were collected, lyophilized, and stored at −20 °C.

Electrophoresis. PAGE-Tricine using a discontinuous gel and buffer system was used for the estimation of the molecular mass of proteins (Schagger and Jagow, 1987).

Proliferation assay. Spleen cells of naïve mice were teased into single-cell suspensions in Hanks' balanced salt solution (HBSS), washed, and suspended in RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM ℓ-glutamine, 1 mM sodium pyruvate, penicillin–streptomycin (Flow Lab., USA), 12.5 mM HEPES buffer (pH 7.4), 0.2% $NaHCO_3$, and 4% fetal bovine serum (Hyclone Laboratories, Okla, USA). The cells were cultured, 2×10^5 per well, in 96 well flat-bottom culture plates in the presence of 2.5 μg/ml of Concanavalin A (Con-A). Cells were incubated for 54 h at 37 °C in a humidified atmosphere containing 5% CO_2, then pulsed with 1.0 μCi of ^3H thymidine per well, and harvested 18 h later with a cell harvester (Cambridge Tech., MA, USA). The incorporation of ^3H thymidine was assessed by standard liquid scintillation techniques.

In vitro cultivation of macrophages with crotapotin. Four days prior to the experiment, naïve SJL mice received a peritoneal injection of 0.5 ml peptone 10%; for the experiment, they received an additional injection of RPMI 1640 medium. The contents of the peritoneal cavity were then withdrawn and distributed in 24-well plates for the cultivation of macrophages. These plates were then incubated for 60 min at 37 °C in an atmosphere containing 5% CO_2 to form adherent macrophage layers before the addition of RPMI 1640 medium and crotapotin with or without the presence of 5 μg/ml lipopolysaccharide (LPS). These plates, as well as control plates without the presence of crotapotin, were again incubated for 24 h. The supernatant was then removed for prostaglandin E_2 determination.

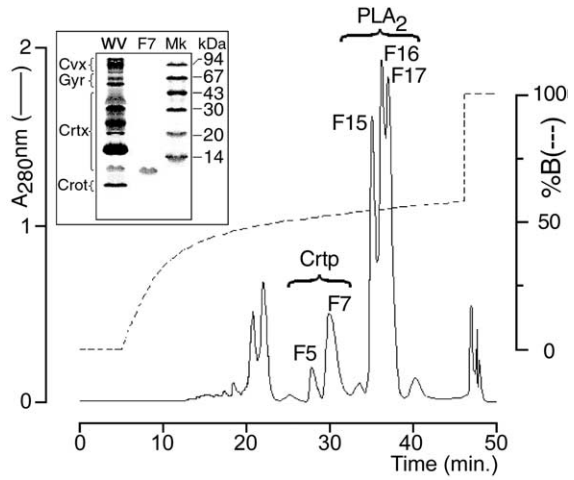
PGE_2 determination. A PGE_2 quantification kit of R&D Systems (MN, USA) was used to assay the presence of PGE_2 in a process based on the competitive binding technique in which the PGE_2 present in a sample competes with a fixed amount of alkaline phosphatase-labeled PGE_2 for sites on a mouse monoclonal antibody which, during incubation, binds to the goat anti-mouse antibody coating the microplate. After washing to remove the excess conjugated and unbound sample, a substrate solution was added to the wells to determine bound enzyme activity. Immediately following color development, the absorbance was read at 405 nm, with intensity of the color inversely proportional to the concentration of PGE_2 in the sample.

Statistical analysis. The statistical significance of the results was determined by a Mann Whitney U test, with a p value smaller than 0.05 considered to be significant.

3. Results

3.1. Purification of C. durissus terrificus venom by RP-HPLC

The crotapotin was isolated from the *C. durissus terrificus* venom through a single RP-HPLC procedure. Fig. 1 shows the seven major fractions, identified as F2, F3, F5, F7, F15, F16, and F17, that were isolated from the whole venom. Mass spectrometry (results not shown) and electrophoresis revealed that the crotapotin isoforms corresponded to fractions F5 and F7. These fractions were



Cvx: Convulxin, Gyr: gyroxin, Crtx: crotoxin, Crot: crotapotin

Fig. 1. Results of RP-HPLC purification of venom from the *C. durissus terrificus* showing isoforms of crotapotin (F5 and F7) and PLA_2 (F15, F16, and F17). The elution of samples was made using a discontinuous buffer B gradient from 0 to 100% of B, with protein absorbance measured at A280 nm. Electrophoresis of F7 to show purity is also shown.

then purified from the venom using RP-HPLC and found to have retention times corresponding to 46 and 49% of Solvent B.

3.2. Crotapotin modification of lymphocyte proliferative response

The effects of crotapotin (fraction F7) on the in vitro proliferative response to Con-A in stimulated spleen cells from naïve mice are shown in Fig. 2. Crotapotin added to the culture at concentrations of 4, 6, and 35 μg/ml effectively inhibited the T cell response to Con-A in a dose dependent manner. Moreover this suppressive effect of crotapotin on lymphocyte proliferation was found to be countered by the addition of indomethacin, an inhibitor of prostaglandin synthesis (Fig. 2). The reduction of cellular growth brought about by 6 μg/ml of crotapotin was partially reversed by indomethacin; moreover, the decrease of the blastogenic response to Con-A in the presence of 4 μg/ml of F7 was completely reversed by this inhibitor of prostaglandin synthesis. However, these effects were not observed for the concentration of 35 μg/ml of crotapotin. Eight groups of experiments were done with three mice in each group.

3.3. PGE_2 production in the supernatant of lymphocyte culture

The presence of PGE_2 in the supernatant of the lymphocyte culture was determined using an ELISA assay. Fig. 3 shows the increase in the production of PGE_2 when lymphocytes were cultured in the presence of crotapotin in concentrations of 2, 4, 6, and 35 μg/ml (133.58, 219.73, 236.84, and 264.47 pg/ml, respectively) as well as its presence in cells cultured in the absence of crotapotin (120.89 pg/ml). These data reinforce the hypothesis that PGE_2 production was induced by crotapotin.

3.4. In vitro crotapotin stimulation of PGE_2 synthesis by peritoneal macrophages

Macrophages from the peritoneum of mice were purified by adherence to plastic plates, a procedure resulting in a preparation of 94% pure macrophages (data not shown). These purified macrophages (10^6 per ml) were cultured in 24-well plates in the presence and absence of LPS (5 μg/ml), as well as that of 2, 6, and 35 μg/ml of crotapotin. After 24 h of incubation, the levels of PGE_2 were determined using an ELISA assay. The stimulation of macrophages with LPS

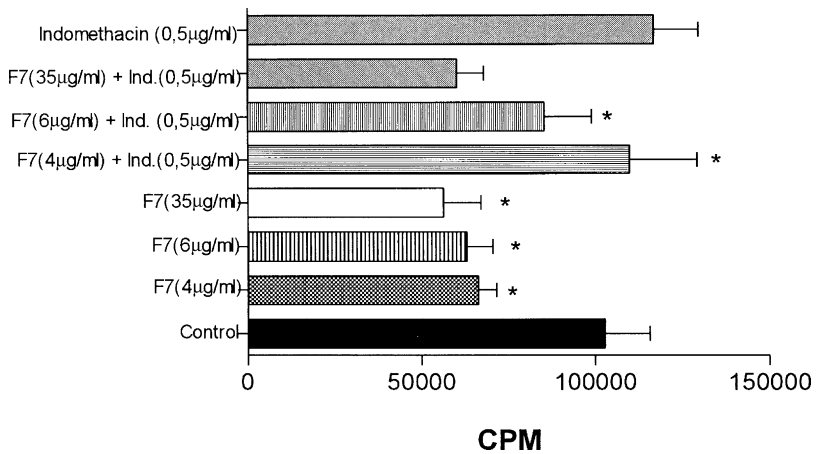


Fig. 2. Effects of crotapotin on in vitro proliferative response of Con-A-stimulated spleen cells (2.5 μg/ml) from naïve mice are shown. The reversal of these effects from addition of indomethacin. The proliferative response was evaluated by ^3H thymidine uptake.

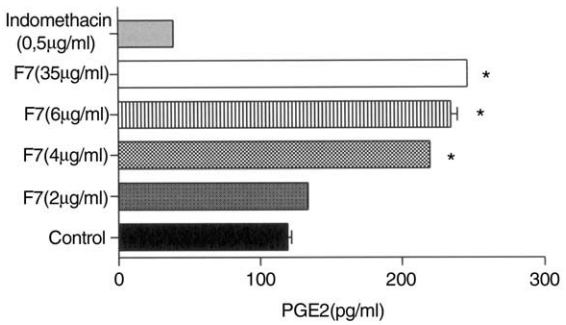


Fig. 3. Levels of PGE_2 produced in the supernatant of lymphocytes cultured in the presence of different concentrations of crotapotin. Results are expressed as means and standard deviation. $* P < 0.01$.

resulted in an increase in the production of PGE_2: 2,690 versus 103.73 pg/ml in the presence and absence of LPS, respectively. The addition of crotapotin to the culture increased the production of PGE_2, whether or not it was previously stimulated with LPS (5,140 and 1000 pg/ml in the presence of 35 µg/ml of crotapotin with and without the presence of LPS, respectively, versus 2,690 and 103.73 pg/ml for the controls with and without LPS) (Fig. 4).

4. Discussion

In the present study, crotapotin has been shown to suppress lymphocyte transformation when added in vitro to the cultures. The addition of indomethacin to the culture abrogated the suppressive effect caused by crotapotin, thus implicating PGE_2 in this suppression. The increased amounts of PGE_2 observed in the supernatant of a culture of lymphocytes and purified macrophages suggested these latter cells as a major source of PGE_2. The early effects of crotapotin suggest the activation of the innate arm of the immune system,

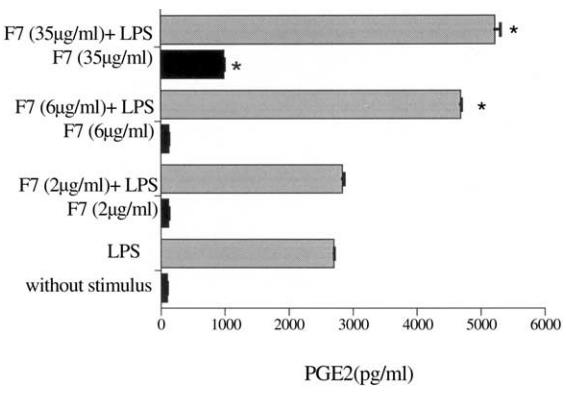


Fig. 4. Average levels of PGE_2 produced in the supernatant of macrophages stimulated or not with LPS (5 µg/ml) in the presence or not of crotapotin ($*$) $P < 0.01$.

and the activation of monocyte/macrophages in this system is known to stimulate the production of a variety of cytokines such as IL1, IL6, TNFα, TGFβ, IL10, and IL12, as well as AA metabolites that promote either activation or suppression of the proliferative response of lymphocytes (Santos et al., 1985; Musatti et al., 1987). The macrophages activated in the innate phase of the immune response will control the clonal selection of lymphocytes bearing antigen receptors, thus ensuring that lymphocytes specific for a given antigen will be activated and clonally expanded in the adaptative immune response. Studies with infectious agents have revealed a dichotomy of CD4 T cell responses, resulting in the production of either T helper 1 (Th1) cells or T helper 2 (Th2) cells, the former predominating in the original presence of IL12 (Hsieh et al., 1993; Manetti et al., 1993) and the latter in the presence of IL4 (Seder et al., 1993; Heinzel et al., 1989).

The mechanisms by which the crotapotin induces an increase in PGE_2 production remain to be clarified, but due to the liaison to its PLA_2, we can infer that this fraction of the venom is acting in this way. Recently, it has been demonstrated that the augment in the production of PGE_2 by macrophages is related the action of PLA_2 as well as that of microsomal PGE synthase. Moreover, the PGE_2 produced by macrophages strongly suppressed the production of macrophage-derived IL12 and T cell-derived IFNγ, suggesting the mediator effect of the PGE_2 on inhibition of Th1 type cytokine (Kuroda and Yamashita, 2003).

Further studies are necessary, but the data already available suggest that whole venom suppresses the lymphocytes of the Th1 subset. The immunosuppressive property of whole venom was previously demonstrated since it induced a significant decrease in the levels of the anti-OVA antibody (Cardoso and Mota, 1997), accompanied by an increase in the production of IL6, IL10, and corticosterone (Cardoso et al., 2001).

As a whole, this paper presents evidence that crotapotin acts as a pro-inflammatory agent that stimulates macrophages to produce PGE_2, consequently reducing the proliferative response of lymphocytes. It is clear that this protein is not inactive and its pharmacological properties deserve further study.

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