UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR

ESTUDO ESTRUTURAL DOS COMPONENTES DO SISTEMA NERVOSO ENTÉRICO E DE CÉLULAS INFLAMATÓRIAS: UMA CONTRIBUIÇÃO À IMUNOPATOLOGIA DO MEGACÓLON CHAGÁSICO

Alexandre Barcelos Morais da Silveira

Belo Horizonte
2007

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UMA CONTRIBUIÇÃO À IMUNOPATOLOGIA DO MEGACÓLON CHAGÁSICO

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Tese apresentada à Pós-Graduação em Biologia Celular do Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Doutor em Biologia Celular

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ICB - UFMG
Belo Horizonte
2007

Este trabalho foi desenvolvido no laboratório do Sistema Linfóide do Departamento de Morfologia, Instituto de Ciências Biológicas, UFMG e no laboratório do Sistema Nervoso Entérico, do departamento de Anatomia e Biologia Celular da Universidade de Melbourne, Austrália.

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ÓRGÃOS FIANCIADORES: CNPq, CAPES e National Health and Medical Research Council of Australia (grant 400020)

Belo Horizonte

2007

Dedico este trabalho à minha esposa Michelle e à minha mãe Anice, que estiveram ao meu lado em todos os momentos. Agradeço por sonharem comigo os meus sonhos e por me concederem o amor e os ensinamentos que foram necessários para que eu os realizasse.

AGRADECIMENTOS

A Deus, meu melhor amigo, pelo amor incondicional, pela força e coragem para enfrentar os momentos difíceis, e por me permitir conhecer pessoas maravilhosas que tornaram mais agradável essa caminhada.

À Dra. Débora d'Ávila Reis por ter me recebido em seu laboratório e transformado um fisioterapeuta recém formado em um pesquisador. Obrigado pelo exemplo como profissional e como ser humano. Graças a sua paciência, empenho e dedicação nós chegamos aqui! Obrigado.

Aos meus amigos Henrique, Luiz Raposo, Alan, Marcelo, Claudinei, Raquel e Diana pelos bons momentos, pelas longas horas de conversa, pela convivência e pelo carinho.

Aos professores da Pós-Graduação em Biologia Celular do Departamento de Morfologia do ICB pelas sugestões, conselhos e ensinamentos.

Ao Dr. John B. Furness pela especial colaboração neste trabalho e pela agradável estadia em Melbourne.

Ao Dr. Enio Oliveira e a Dr. Sheila Adad por acreditarem neste projeto e na minha capacidade de realizá-lo.

A Iraídes pela disponibilidade e competência na realização de procedimentos que facilitaram a realização deste trabalho.

Aos amigos que contribuíram direta e indiretamente.

Aos pacientes estudados neste trabalho pela oportunidade de aprendizado.

"Cada um que passa em nossa vida, passa sozinho, pois cada pessoa é única e nenhuma substitui a outra. Cada um que passa em nossa vida, passa sozinho, mas quando parte, nunca vai só nem nos deixa a sós. Leva um pouco de nós, deixa um pouco de si mesmo".

Saint-Exupéry

Esta tese está apresentada segundo moldes aprovados pelo Colegiado do Curso de Pós-graduação em Biologia Celular do Instituto de Ciências Biológicas da UFMG.

De acordo com o a resolução número 04/2000, de dezembro de 2000, as teses podem ser apresentadas em dois formatos, o tradicional e o formato de compilação de artigos. Como formato de compilação de artigos, os seguintes requisitos mínimos deverão ser obedecidos:

- Para tese, exige-se a publicação de pelo menos dois artigos completos.
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- O aluno deverá ser primeiro autor em pelo menos um dos artigos
- Deverão integrar a tese os seguintes tópicos: introdução, discussão, artigos resultantes, conclusão e perspectivas.

SUMÁRIO

LISTA DE ABREVIATURAS

ATP - adenosina tri-fosfato

ChaT - Colina acetil transferase

CD - *Cluster of diferentiation*

CGRP - Peptídio relacionado ao gene de calcitonina

ECP - Proteína eosinofílica catiônica

EDN - Neurotoxina derivada de eosinófilos

EPO - Peroxidase eosinofílica

GFAP - Proteína acídica fibrilar da glia

H&E - Hematoxilina & Eosina

IL - Interleucina

IPANs - neurônios intrínsecos primários aferentes

MBP - Proteína básica principal

MHC - Complexo principal de histocompactibilidade

NOS - Óxido nítrico sintase

NPY - Neuropeptídeo Y

PCR - Reação em cadeia da polimerase

PGP 9.5 - Proteína gene produzida 9.5

SNA - Sistema nervoso autônomo

SNE - Sistema nervoso entérico

SP - Substância P

T. cruzi - *Trypanosoma cruzi*

TIA-1 - Antígeno intracitoplasmático de células T

TNF-α - Fator de necrose tumoral alfa

VIP - Polipeptídeo intestinal vasoativo

INTRODUÇÃO

Doença de Chagas

Em fins de 1907, encarregado por Oswaldo Cruz, Carlos Chagas viajou para Lassance, arraial próximo às margens do Rio São Francisco, onde a malária devastava o acampamento dos trabalhadores da Estrada de Ferro Central do Brasil. Instalou sua casa e seu laboratório em um vagão de trem. No povoado, observando a infinidade de insetos hematófagos alojados nas paredes de pau-a-pique das moradias, decidiu examiná-los. Encontrou neles um novo parasita, que chamou de *Trypanosoma cruzi*, em homenagem a Oswaldo Cruz, seu amigo e mentor. Verificou que o parasito era patogênico para animais de laboratório e descobriu sua presença em animais domésticos (Koberle, 1957).

A presença deste parasita em insetos sugeriu a possível existência de uma doença infecciosa em animais e no próprio homem. Começou então a pesquisar as ligações entre o novo parasita e a condição mórbida daquela população. Dois anos depois, em agosto de 1911, Carlos Chagas expôs suas descobertas na Academia Nacional de Medicina, no Rio de Janeiro, descrevendo detalhadamente as fases aguda e crônica da doença, além de suas diferentes formas clínicas. Entretanto, tais relatos não foram bem aceitos pela comunidade científica e até mesmo negados por alguns dos seus contemporâneos. Carlos Chagas chegou mesmo a ser denominado de “o homem que procura na selva doenças que não existem”. Após 1920, a doença de Chagas foi simplesmente esquecida e por mais de 10 anos foi considerada sem importância para a saúde pública (Koberle, 1968; Prata, 1999).

Em 1934, Mazza demonstrou vários casos agudos da doença de Chagas no norte da Argentina, exatamente onde outros pesquisadores haviam admitido não haver qualquer pessoa contaminada com o parasita. Da mesma forma que Carlos Chagas, Mazza foi criticado por “descobrir novas doenças ao invés de procurar cura para as que já existiam”. Resistindo aos ataques, ele e sua equipe persistiram em suas investigações, identificando mais de 1000 casos agudos até 1944, comprovando ser a doença de Chagas naquela época um problema de saúde pública. Apesar da “re-descoberta” da doença por Mazza, o significado real da mesma foi reconhecido somente com o aperfeiçoamento da técnica de fixação de complemento e de seu uso

como diagnóstico na população. A partir de então, a doença de Chagas passou a ser denominada de trypanosomiasis americana (Koberle, 1961; Koberle, 1968; Prata, 1999).

Dentre as formas de transmissão da doença de Chagas, destacamos a via vetorial, a via placentária e a via transfusional, sendo esta última, atualmente, a forma mais comum de contágio no Brasil. Quando a contaminação se dá a partir da picada do inseto transmissor, a mesma ocorre através das fezes contaminadas. Após o ato de sugar o sangue, o que ocorre na maioria das vezes durante a noite, o inseto elimina fezes contaminadas com a forma tripomastigota próximo ao local da picada. Os parasitas conseguem penetrar ativamente através da mucosa ou mesmo da conjuntiva ocular e após invadirem as células do hospedeiro, podem escapar dos mecanismos de defesa do organismo. Em seguida, o parasita tem acesso a vasos linfáticos e sangüíneos, indo parasitar uma variedade de células em outros órgãos. Dentro das células, os parasitas diferenciam-se em amastigotas, reproduzem-se e dão origem a novas formas tripomastigotas, as quais retornam à circulação sistêmica, reiniciando o ciclo (Brener, 1982; Koberle, 1968).

Após a infecção os pacientes desenvolvem a fase aguda da doença de Chagas, quando eles podem vir a falecer de miocardite, de meningoencefalite ou de complicações, como broncopneumonia. Em crianças de até cinco anos de vida, os sintomas da infecção aguda são mais severos do que aqueles observados em adultos. A fase aguda representa na realidade uma infecção generalizada pelo *T. cruzi*. As formas tripomastigotas do parasita, não raramente, são encontradas no sangue, enquanto as formas amastigotas são observadas difusamente em células do organismo, incluindo macrófagos, células da glia, células adiposas, células endoteliais, fibras musculares lisa, esquelética e cardíaca, fibroblastos, células de Schwann e neurônios. Nesta fase é comum a presença do sinal de Romaña, ou chagoma, que possui significado de uma lesão de porta de entrada (Dias, 2001; Koberle, 1968; Koberle, 1970).

Na fase crônica, os indivíduos podem apresentar sintomas resultantes do comprometimento do sistema digestivo e/ou cardíaco, ou podem ainda persistir na forma assintomática da doença, também denominada de forma indeterminada. O desenvolvimento ou não das formas sintomáticas da doença na fase crônica representa um dos aspectos mais enigmáticos sobre a doença de Chagas, uma vez que pode haver um intervalo de 20 até 30 anos entre a fase aguda e a fase crônica sintomática. Alguns indivíduos chegam a falecer com 70 a 80 anos sem nunca apresentar qualquer sintoma decorrente da infecção (Koberle, 1968).

A forma crônica cardíaca, pela sua gravidade e freqüência, é uma das formas mais bem estudadas da doença de Chagas. Esta forma leva à insuficiência cardíaca, transtornos do ritmo e da condução, fenômenos tromboembólicos e morte súbita. Pacientes portadores desta forma clínica apresentam miocardite usualmente intensa e difusa, sendo acompanhada de cardiomegalia, lesões vasculares e fibrose (De Rezende & Rassi, 1958; Marin Neto *et al.*, 1980; Rassi *et al.*, 2000).

Pacientes portadores da forma digestiva apresentam sintomas decorrentes de comprometimento de órgãos deste sistema, principalmente do esôfago (megaesôfago) e do cólon (megacólon). Acredita-se que um dos fatores mais importantes no desenvolvimento do mega chagásico seja um processo degenerativo, principalmente de gânglios nervosos do sistema nervoso entérico (SNE), que aparentemente tem seu início na fase aguda, persistindo até a fase crônica (Andrade & Andrade, 1966; Andrade & Andrade, 1969; Koberle, 1968).

A primeira suspeita da existência da forma digestiva na doença de Chagas surgiu em 1916, quando o próprio Carlos Chagas observou que durante a infecção aguda, alguns adultos exibiam uma acentuada disfagia para determinados tipos de alimentos cuja ingestão necessitava de ser acompanhada de água. Os pacientes relatavam que o trânsito do alimento era interrompido no esôfago, causando imensa dor. Mesmo a ingestão de líquidos poderia ser difícil, sendo às vezes impossível, havendo desta forma, a necessidade de que o mesmo fosse administrado em pequenas doses. Tal fenômeno, sem qualquer explicação patogênica na época, foi denominado então de “Mal do Engasgo” (Chagas, 1916).

O megacólon chagásico atinge, sobretudo, o sigmóide e o reto. Pode manifestar-se como uma doença isolada, mas freqüentemente é encontrado associado ao megaesôfago ou à cardiopatia chagásica. É mais comum no adulto (30 a 60 anos) e mais incidente no sexo masculino (Dias, 2001).

Como o primeiro sintoma do megacólon é a constipação, tanto o diagnóstico clínico como o anatômico são em geral tardio, após o instituir da dilatação. À microscopia ótica de luz observam-se: 1) Infiltrados inflamatórios crônicos, focais e difusos na muscular da mucosa, na submucosa e nas camadas musculares; 2) Lesões do SNE, especialmente do plexo mientérico, com periganglionite e ganglionite focais ou difusas. São observados intensos fenômenos regressivos de neurônios, chegando à destruição completa dos gânglios nervosos do plexo mientérico; 3) Ulcerações e inflamação crônica da mucosa, focal ou difusa em casos mais

avançados, podendo atingir a submucosa; 4) Fibrose intermuscular, focal ou difusa e fibrose de substituição (Campos & Tafuri, 1973; Tafuri, 1970; Tafuri, 1987; Tafuri & Brener, 1967). Alterações ultra-estruturais do plexo mientérico consistem em lesões, em geral focais, de todos os componentes dos gânglios: neurônios, células de Schwann e fibras nervosas. Por isso, é comum, no mesmo gânglio, a existência de neurônios, às vezes, profundamente lesados ao lado de outros morfologicamente íntegros (Tafuri, 1971; Tafuri *et al.*, 1971).

Segundo Tafuri *et al.* (1971), é lícito admitir uma progressividade das lesões dos plexos, que se agravam proporcionalmente à duração e ao grau do mega. O acúmulo de fezes no cólon provoca dilatação da luz e compressão da mucosa. A compressão, por sua vez, leva a isquemia, e secundariamente, a degeneração, necrose e ulceração da mucosa. Na mucosa assim ulcerada inicia-se um processo inflamatório secundário e independente da inflamação induzida pela própria doença de Chagas. Esse processo inflamatório atinge o plexo mientérico já previamente lesado pelo *T. cruzi*, agravando ainda mais a destruição do SNE. Por sua vez, o plexo submucoso sofre as conseqüências das lesões do plexo mientérico, devido às relações sinápticas entre eles. A inflamação secundária à estase somada à destruição dos plexos e dos componentes intersticiais evolui para a fibrose da submucosa e do conjuntivo intermuscular. Com o tempo ocorrem hipertrofia e alterações regressivas das fibras musculares. Como o plexo submucoso está em íntima relação com as células musculares é fácil compreender como a miosite e suas seqüelas podem lesar ainda mais os gânglios.

A escassez de parasitas em relação à intensidade e à extensão das lesões na fase crônica da doença, levaram diversos autores a avaliar o envolvimento de fatores autoimunes na patogênese da lesão chagásica. Alguns autores relataram a existência de reação cruzada entre componentes autólogos e antígenos do *T. cruzi* (Al-Sabbagh *et al.*, 1998; Cunha-Neto *et al.*, 1995; Levitus *et al.*, 1991). Estudos utilizando modelo de infecção experimental pelo *T. cruzi* sugerem que durante a fase aguda da infecção, haveria uma ativação policlonal responsável pela liberação de clones auto-reativos que persistiriam por longos períodos no hospedeiro, levando ao surgimento das lesões (d'Imperio Lima *et al.*, 1986; Minoprio *et al.*, 1986a; Minoprio *et al.*, 1986b).

Embora o parasitismo seja escasso em relação à intensidade e à extensão das lesões, vários estudos não deixam dúvida quanto à presença do parasita nos tecidos de pacientes chagásicos. Almeida *et al.* (1984) verificaram que o processo inflamatório cardíaco era

particularmente evidenciado em células musculares parasitadas. Barbosa & Andrade (1985) demonstraram através de autópsias de pacientes com miocardite chagásica difusa, a presença de formas amastigotas de *T. cruzi* em amostras de coração, bem como de tecidos extra-cardíacos. Com a utilização de anticorpos policlonais anti-*T. cruzi* em tecidos de coração de pacientes com cardiopatia chagásica, Higuchi *et al.* (1993) demonstraram existir uma estreita correlação entre a presença de antígenos do parasita e a intensidade do infiltrado inflamatório. Outra metodologia utilizada foi a técnica de reação em cadeia da polimerase (PCR), através da qual é detectado o kDNA de *T. cruzi* em lesões inflamatórias de pacientes com cardiopatia chagásica e de chagásicos com megaesôfago (da Silveira *et al.*, 2005b; Jones *et al.*, 1993; Vago *et al.*, 1996). O kDNA do parasita é também encontrado no cólon de pacientes portadores de megacólon chagásico, sendo mais freqüente na porção entre o reto e o sigmóide (dados não publicados do nosso grupo de pesquisa).

O processo inflamatório na fase crônica da doença de Chagas apresenta sempre sinais de atividade celular. No megaesôfago chagásico, os infiltrados inflamatórios são compostos de 72-93% de linfócitos T $CD3^+$, de 6-29% de macrófagos $CD68^+$ e 1-4% de linfócitos B $CD20^+$. Cerca de 1-35% das células do infiltrado inflamatório nas camadas musculares expressam TIA-1 (antígeno intracelular de célula T) uma proteína encontrada em linfócitos T citotóxicos e células *Natural Killer* (d'Avila Reis *et al.*, 2001). Linfócitos T citotóxicos produtores de granzima A e células *Natutal Killer* foram também demonstrados em lesões do coração de pacientes portadores de cardiopatia chagásica (Reis *et al.*, 1993). No cólon de pacientes portadores de megacólon, Corbett *et al.* (2001) demonstraram a presença de células *Natural Killer*, sugerindo assim a participação destas na continuidade do processo inflamatório da fase crônica.

Lemos *et al.* (1998) estudando pacientes que apresentavam a forma digestiva da doença de Chagas, realizaram análise do sangue destes indivíduos com o objetivo de verificar o fenótipo dos linfócitos circulantes. Foi observada uma diminuição significativa do número de linfócitos T $CD3/CD4^+$ e de linfócitos B $CD19^+$. A razão "número de linfócitos T $CD4^+$ / número de linfócitos T $CD8^+$" apresentou-se diminuída em indivíduos portadores de megaesôfago avançado, demonstrando assim um decréscimo mais significativo do número de linfócitos T $CD4^+$, o que não é observado em pacientes chagásicos cardiopatas, não portadores de mega (Dutra *et al.*, 1994). Como marcador de ativação de linfócitos T em pacientes portadores de mega, foi utilizado um anticorpo anti-HLA-DR e desta forma demonstrou-se elevação dos níveis de linfócitos T

ativados, o que já havia sido observado em pacientes portadores de cardiopatia chagásica ou mesmo assintomáticos (Dutra *et al.*, 1994). Ainda em pacientes portadores de mega, verificou-se uma queda da porcentagem de células CD4/CD28^{+}, o que poderia sugerir uma falha em mecanismos de resistência imunológica e de alguma forma contribuir para a progressão da doença (Lemos *et al.*, 1998).

Os eosinófilos são células presentes em grande número no sangue e tecidos de pacientes chagásicos da fase crônica. Nakhle *et al.* (1989), utilizando camundongos infectados com *T. cruzi*, avaliaram a cinética de liberação de eosinófilos pela medula óssea, sugerindo um papel para estas células no processo de resistência ao parasita. Molina e Kierszenbaum realizaram uma série de estudos nos quais foram demonstradas associações entre eosinófilos e algumas alterações patológicas associadas à infecção. No miocárdio de pacientes chagásicos, esses autores demonstraram a presença de depósitos de uma neurotoxina derivada de eosinófilos (Molina & Kierszenbaum, 1988a), bem como a presença de eosinófilos ativados (Molina & Kierszenbaum, 1989a). Foi também demonstrada uma correlação entre concentração de eosinófilos e severidade das lesões inflamatórias no miocárdio e musculatura esquelética (Molina & Kierszenbaum, 1988b). O papel deste granulócito na lesão de cardiomiócitos infectados com *T. cruzi* foi também sugerido a partir de estudos *in vitro*, com co-cultura de cardiomiócitos e eosinófilos (Molina & Kierszenbaum, 1989b).

Uma outra célula do sistema imune que possui papel relevante na evolução da doença de Chagas é o mastócito e o seu papel nesta patologia já foi alvo de vários estudos. Almeida *et al.* (1989), trabalhando com ratos infectados com *T. cruzi*, evidenciaram no estômago destes animais redução dos níveis de acetilcolina e aumento dos níveis de histamina e do número de mastócitos na parede gástrica. Pires *et al.* (1992) observaram aumento dos níveis de histamina em vários órgãos de camundongos infectados por *T. cruzi*, indicando que mastócitos estariam realizando um importante papel no processo inflamatório. Pinheiro *et al.* (1992), analisando ratos Wistar na fase aguda da infecção por *T. cruzi* verificaram no miocárdio aumento da concentração de mastócitos associados aos focos inflamatórios. Postan *et al.* (1994) através de estudos *in vitro*, sugeriram que a presença de mastócitos esteja diretamente relacionada ao desenvolvimento de fibrose em cardiomiócitos infectados pelo *T. cruzi.*

Acreditamos na existência de uma interconexão entre sistema imune e neuro-endócrino. Essa ligação promoveria uma troca de informações bi-direcional entre sistema imune e sistema

nervoso entérico. A ativação de mastócitos, além de atuar na fisiologia gastrintestinal, desempenha um papel crucial no processo inflamatório, sendo um dos principais codificadores de sinais intestinais que irão culminar em respostas motoras, percepções viscerais e ativação de células do sistema imunológico em patologias gastrintestinais (Gui, 1998), e, possivelmente também na doença de Chagas.

O Sistema Nervoso Entérico

A inervação das vísceras digestivas é extremamente complexa. Existem cerca de 80 a 100 milhões de neurônios dispersos ou reunidos em pequenos gânglios ou em dois plexos interconectados (plexo mientérico e plexo submucoso). Nesses plexos são grandes as variedades de tipos neuronais, seus inúmeros neurotransmissores e receptores e, portanto, diversas as propriedades funcionais, constituindo uma verdadeira rede de controle da motilidade digestiva e vascular. Essa surpreendente complexidade e variedade morfofuncional levaram os fisiologistas a proporem a existência de um sub-sistema do sistema nervoso autônomo (SNA), denominado de sistema nervoso entérico (SNE) (Furness & Costa, 1983).

Funcionalmente, a maior parte dos neurônios encontrados no plexo mientérico são neurônios eferentes (Gabella & Trigg, 1984). A inervação das camadas musculares se dá através de projeções de feixes nervosos provenientes do plexo mientérico. Estes feixes estão dispostos paralelamente às fibras musculares (Richardson, 1958).

O plexo submucoso, descrito por Meissner (Meissner, 1857) e Billroth (Billroth, 1858), assim como o plexo mientérico, são formados de gânglios interconectados por feixes nervosos, O plexo submucoso forma uma rede contínua em torno da circunferência e por todo trato gastrintestinal (Figura 1), onde duas ou três camadas de gânglios podem ser observadas (Gunn, 1968; Hoyle & Burnstock, 1989; Schabadasch, 1930). Entre os neurônios encontrados no plexo submucoso externo, alguns inervam a camada muscular interna e ocasionalmente a muscular externa (Furness *et al.*, 1990; Porter *et al.*, 1999; Sanders & Smith, 1986).

O efeito do sistema nervoso simpático no trato gastrintestinal é mediado principalmente pela noradrenalina, que é liberada por seus axônios pós-ganglionares. Os corpos destes neurônios encontram-se nos gânglios nervosos pré-vertebrais e paravertebrais, enquanto seus axônios

conectam-se ao trato gastrintestinal através dos nervos mesentéricos. Quando estimulados, estes neurônios agem inibindo a peristalse, regulando o fluxo sanguíneo dos vasos intestinais e controlando a secreção de eletrólitos (Costa *et al.*, 2000; Lundgren, 2000; McMillin *et al.*, 1999; Powley, 2000b).

O sistema nervoso parassimpático atua no trato gastrintestinal através dos nervos vago e pélvicos. O nervo vago origina-se de corpos neuronais localizados no encéfalo, enquanto os nervos pélvicos possuem seus corpos neuronais na coluna intermédio-lateral sacra. Os estímulos vagais utilizam acetilcolina como neurotransmisor, sendo esta responsável por estimular a peristalse e aumentar o aporte sanguíneo intestinal (Powley, 2000a).

Com a evolução das técnicas imunohistoquímicas, a riqueza de neuromediadores do SNE começou a ser revelada. O desenvolvimento da técnica de fluorescência por Falck (Falck, 1962) permitiu que autores como Norberg (Norberg, 1964) identificassem com exatidão os axônios terminais de neurônios simpáticos pós-ganglionares. Os neurônios colinérgicos entéricos foram identificados pela técnica de imunohistoquímica somente no início da década de 80 (Furness *et al.*, 1983). Desde as primeiras descrições de Hokflef e sua equipe (Hökfelt *et al.*, 1975) sobre a presença de somatostatina em neurônios entéricos, a localização de outros neuropeptídeos tornou-se alvo de inúmeros estudos (Costa & Furness, 1982; Furness *et al.*, 1980; Schultzberg *et al.*, 1980; Sundler *et al.*, 1980). A partir destes estudos, foi revelada a presença de neuropeptídeo Y (NPY), substância P (SP), peptídeo intestinal vasoativo (VIP) e serotonina em neurônios entéricos. Foi ainda demonstrada a co-existência de neuropeptídeos nos mesmos grupos neuronais, tanto do SNC quanto do SNE, (Hokfelt *et al.*, 1984; Hokfelt *et al.*, 1980), marcando o início de uma nova era nos estudos da codificação neuroquímica de neurônios.

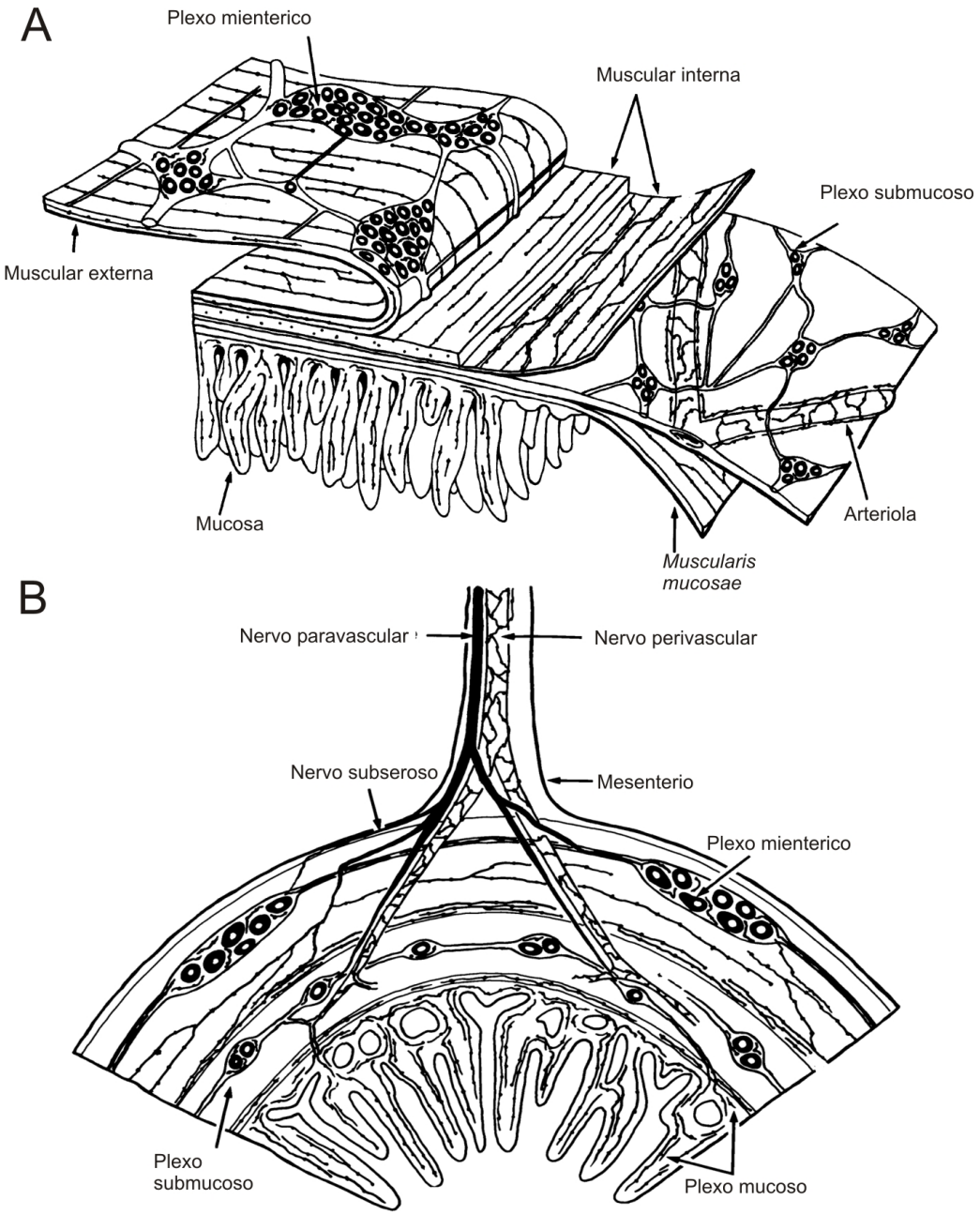

***Figura 1**: Esquema do SNE observado em camadas (A) e em secção transversal (B). Existem dois plexos nervosos formados por gânglios; o plexo mientérico e o plexo submucoso, além das fibras nervosas que inervam as camadas musculares, a mucosa e as arteríolas intramurais. A inervação extrínseca tem acesso ao SNE através de nervos paravasculares e perivasculares (B). Adaptado de Furness e Costa (Furness & Costa, 1980), com permissão dos autores.*

Com relação ao SNE humano, alguns trabalhos se destacaram ao estabelecer correlações entre a neuroquímica, a morfologia e a fisiologia neuronal. Em 2004, Brehmer e sua equipe (Brehmer *et al.*, 2004a), através do uso de marcadores para neurofilamentos, realizaram a descrição morfológica dos neurônios intestinais de indivíduos de diferentes faixas etárias. Neste mesmo ano foi realizada a caracterização imunoquímica dos neurônios intrínsecos do intestino delgado (Brehmer *et al.*, 2004b). Em 2005, este autor descreveu a morfologia de neurônios do plexo mientérico imunoreativos a encefalina, VIP e NOS (óxido nítrico sintase) (Brehmer *et al.*, 2005). Esses trabalhos mostram que a complexidade neuroquímica do SNE humano é ainda maior do que se imaginava e suscitam a necessidade da realização de mais trabalhos que visem a sua caracterização, principalmente em patologias gastrintestinais nas quais, segundo Camilleri (Camilleri, 2001), o perfil neuroquímico do SNE estaria alterado.

Os neurônios entéricos podem ser classificados funcionalmente como neurônios motores, interneurônios e neurônios intrínsecos primários aferentes (IPANs). Os neurônios motores podem ser divididos em dois grupos, os excitatórios e os inibitórios. Ambos inervam as túnicas musculares e a muscular da mucosa em todo trato gastrintestinal. Os principais neuromediadores encontrados nos neurônios excitatórios são a acetilcolina e as taquicininas. Os neurônios inibitórios possuem vários neuromediadores, como NO (óxido nítrico), VIP e adenosina tri-fosfato (ATP) (Furness *et al.*, 1995b).

Os interneurônios são identificados em todas as camadas do trato gastrintestinal, sendo que sua constituição neuroquímica varia muito, dependendo do órgão em questão. Por exemplo, os neurônios motores e os neurônios aferentes do íleo e do cólon expressam basicamente os mesmos neuromediadores, o que não é verdade quando se tratam de interneurônios (Furness *et al.*, 1995a).

Os IPANs, por alguns denominados de neurônios sensoriais, traduzem e codificam informações sobre o ambiente químico e estado físico do tecido que eles inervam, e transmitem essa informação para um circuito neuronal integrado, através do qual o estado funcional do órgão pode ser modificado. A denominação IPANs deve-se ao fato destes neurônios exercerem, em algumas situações, papeis funcionais de interneurônios (por exemplo, quando recebem sinapses excitatórias provenientes de outros neurônios) e mesmo de neurônios eferentes (por exemplo, quando liberam neurotransmissores na mucosa causando vasodilatação) (Holzer *et al.*, 1991;

Lewis, 1927). Nos plexos mientérico e submucoso, esses neurônios se conectam a outros IPANs, a interneurônios e a neurônios motores (Dogiel, 1899; Gershon & Kirchgessner, 1991).

Evidências recentes indicam que os IPANs são influenciados por processos inflamatórios, tanto no intestino delgado como no cólon. Em patologias inflamatórias intestinais, como doença de Chron e colite ulcerativa, as propriedades funcionais dos IPANs são modificadas, alterando conseqüentemente a sinalização sensorial e o controle dos reflexos entéricos (Sharkey & Mawe, 2002). Em modelos experimentais foi demonstrado que um dos mediadores inflamatórios envolvidos neste processo são as prostaglandinas (Manning *et al.*, 2002; Palmer *et al.*, 1998).

Os neuromediadores do SNE possuem atividade considerável sobre o sistema imune. A substância P, por exemplo, é considerada uma substância pró-inflamatória. Ela estimula a proliferação linfocitária, o tráfego de linfócitos através dos linfonodos e a produção de IL-2. Além disso, a substância P age como um dos ativadores de células *Natural Killer* e mastócitos e possui ação quimiotática para monócitos e neutrófilos. Já o neuropeptídeo VIP inibe a resposta de células *Natural Killer* e de linfócitos T, bem como a produção de IL-2 e IL-4 por estas células. Por outro lado, VIP estimula a quimiotaxia de monócitos e a produção de IL-5 por linfócitos (McKay & Fairweather, 1997).

O sistema imune, por sua vez, também influencia atividades do SNE através da secreção de vários tipos de substâncias, como por exemplo, as neurotrofinas. As neurotrofinas são grupos heterogêneos de polipeptídeos que, através de seus receptores específicos, exercem papel essencial no desenvolvimento, diferenciação, sobrevivência, manutenção e regeneração do sistema nervoso (Barbacid, 1995; Griesbeck *et al.*, 1999; Lo, 1992; Roux & Barker, 2002).

Outro componente que participa da fisiologia do trato gastrintestinal, juntamente com os neurônios e as células do sistema imune, são as células da glia entérica. As células da glia entérica, ou células enterogliais, são muito semelhantes aos astrócitos encontrados no SNC. Elas expressam a proteína estrutural S-100 (Ferri *et al.*, 1982) e apresentam também, em certas situações, a proteína acídica fibrilar da glia (GFAP) (Jessen & Mirsky, 1983). Células enterogliais possuem receptores para citocinas e também são capazes de produzir algumas delas, como por exemplo, a IL-6. Além disso, elas possuem receptores para neurotransmissores, os quais podem modular a expressão de citocinas pelas mesmas. Como pode ser notado, as células enterogliais representam um elo de comunicação entre o SNE e o sistema imune local, possuindo assim um papel relevante na fisiologia intestinal (Ruhl *et al.*, 2004).

Bush *et al.* (1998) depletaram camundongos adultos de células $GFAP^+$ para avaliar a importância das mesmas na fisiologia intestinal e observaram que, em apenas duas semanas, todos os animais morreram devido a um quadro de jejunoileíte fulminante. Esse quadro foi independente de processos infecciosos, sendo o mesmo caracterizado por degeneração de neurônios mientéricos e hemorragia intestinal. Esses dados confirmam o papel da glia entérica como mantenedora da integridade intestinal.

Von boyen *et al.* (2004) demonstraram que, sob influência de citocinas pró-inflamatórias, células enterogliais $GFAP^-$ podem se tornar $GFAP^+$. O aumento da expressão de GFAP por células enterogliais é também observado em tecidos coletados de pacientes portadores de colite ulcerativa e doença de Crohn. Estudos sobre esta patologia têm confirmado que a lesão de células do SNE é caracterizada por severa diminuição do número de células da glia, mesmo em tecidos sem evidência de processo inflamatório. Uma significativa redução de células da glia tanto do plexo mientérico, como do plexo submucoso é também uma das características histopatológicas da enterocolite necrosante (Cornet *et al.*, 2001).

Na doença de Chagas, como resultado de lesões de estruturas do SNE, verifica-se distúrbio do peristaltismo, falta de coordenação motora, acalasia do esfíncter, retenção de fezes no reto e cólon sigmóide, hipertrofia muscular e finalmente, dilatação, levando ao aparecimento do megacólon chagásico (de Rezende, 1979; Tafuri *et al.*, 1971). Dados provenientes de estudos realizados sobre a forma digestiva da doença de Chagas sugerem que a destruição neuronal observada tanto no megaesôfago e megacólon chagásico possui relação estreita com a intensidade do processo inflamatório e com a evolução da patologia (Adad *et al.*, 1991; Adad *et al.*, 2001; da Silveira *et al.*, 2005b). No entanto, estudos com uma abordagem mais ampla, relacionando as alterações sofridas pelos componentes do SNE e as células inflamatórias observadas no megacólon chagásico, ainda são escassos na literatura.

Segundo Koberle, para o desenvolvimento do megaesôfago é necessário que haja uma redução de neurônios no órgão de cerca de 85%, enquanto a doença no cólon está associada a uma perda neuronal de no mínimo 50% (Koberle, 1968). Em nosso trabalho de mestrado observamos que alguns pacientes, não portadores de megaesôfago e sem qualquer sintoma digestivo, apresentaram uma redução neuronal no esôfago de aproximadamente 90%, ultrapassando assim o limite estabelecido anteriormente por Koberle. É possível que, para o desenvolvimento do mega chagásico, seja relevante não somente a taxa de destruição neuronal,

mas também a seletividade desse processo, o que levaria a perda de determinados tipos funcionais de neurônios em detrimento de outros, ocasionando assim distúrbios no peristaltismo e conseqüente desenvolvimento do megaesôfago ou megacólon.

Neste trabalho, tivemos por objetivo dar continuidade à linha investigativa de nosso laboratório sobre a imunopatologia da forma digestiva da doença de Chagas. Trata-se de um estudo descritivo das células inflamatórias, das células da glia e dos neurônios encontrados em pacientes chagásicos, portadores e não portadores de megacólon. A partir dos quadros encontrados e da revisão de literatura aqui apresentada, algumas hipóteses sobre o desenvolvimento do megacólon chagásico foram levantadas.

OBJETIVOS

Objetivo geral

Caracterizar e quantificar células inflamatórias presentes no cólon de pacientes chagásicos, portadores e não portadores de megacólon, e verificar a co-relação entre as mesmas e as alterações funcionais e estruturais ocorridas no SNE, visando contribuir para a compreensão da patologia do megacólon chagásico.

Objetivos específicos

1. Avaliar comparativamente, em pacientes chagásicos portadores e não portadores de megacólon, os seguintes parâmetros abaixo:

a) Fenótipo e distribuição das células inflamatórias encontradas nas camadas musculares e plexos nervosos do cólon. Para isso utilizamos a técnica de imunohistoquímica com os seguintes anticorpos: anti-CD3 (linfócitos T), anti-CD20 (linfócitos B), anti-TIA-1 (linfócitos T citotóxicos), anti-CD57 (células *Natural Killer*), anti-CD68 (macrófagos). Para análises de eosinófilos e mastócitos utilizamos a colorações com hematoxilina & eosina e azul de toluidina, respectivamente.

b) Área de fibrose.

c) Intensidade de desnervação, através da quantificação de filetes nervosos PGP 9.5^{+} nas camadas musculares do cólon.

d) Fenótipo das células enterogliais. Para isso utilizamos um marcador pan-glial (anti-S-100) e um marcador para células da glia sob processo inflamatório (anti-GFAP), ambos empregados na técnica de imunohistoquímica.

e) A co-relação entre densidade de filetes nervosos PGP 9.5^{+} e concentração de leucócitos com potencial citotóxico (linfócitos TIA-1, *células Natural Killer*, eosinófilos e macrófagos).

f) A co-relação entre área de fibrose e as concentrações de eosinófilos, mastócitos e macrófagos.

g) A co-relação entre a presença de células enterogliais e a densidade de filetes nervosos PGP 9.5^{+}.

2. Verificar se existe uma destruição seletiva de neurônios nos plexos nervosos do cólon de pacientes chagásicos portadores de megacólon. Para isso avaliamos a expressão dos seguintes marcadores neuroquímicos: calretinin (IPANs - neurônios sensoriais), ChAT e substância P (neurônios motores excitatórios), NPY (interneurônios), VIP e nNOS (neurônios motores inibitórios).

RESUMO DOS RESULTADOS

1. Os focos inflamatórios presentes nas camadas musculares e nos plexos nervosos do cólon no cólon de pacientes chagásicos portadores e não portadores de megacólon são constituídos de linfócitos T $CD3^+$, linfócitos T $CD8^+$, linfócitos B $CD20^+$, linfócitos T citotóxicos TIA-1^+, células *Natural Killer* $CD57^+$, macrófagos, eosinófilos e mastócitos (Anexo 1).

2. Em ambos grupos de pacientes chagásicos, a célula predominante no plexo mientérico é linfócito T $CD3^+$, enquanto no plexo submucoso a célula predominante foi o linfócito B $CD20^+$ (Anexo 1).

3. Pacientes chagásicos portadores de megacólon apresentam uma quantidade estatisticamente maior de células inflamatórias citotóxicas (linfócitos citotóxicos TIA-1^+, células *Natural Killer* $CD57^+$, macrófagos e eosinófilos) e mastócitos em relação aos pacientes chagásicos não portadores de megacólon (Anexo 1 e 2).

4. Pacientes chagásicos portadores de megacólon apresentam uma área de fibrose significativamente maior que aquela apresentada por pacientes chagásicos não portadores de megacólon (Anexo 2).

5. Existe uma co-relação direta entre área de fibrose e concentração de eosinófilos, mastócitos e macrófagos nas camadas musculares do cólon de pacientes chagásicos (Anexo 2).

5. Indivíduos chagásicos portadores de megacólon apresentam uma área de filetes nervosos PGP 9.5^+ nas camadas musculares significativamente menor que aquela apresentada por pacientes chagásicos não portadores de megacólon (Anexo 1).

6. Pacientes chagásicos apresentam um número de células enterogliais S-100^+ significativamente menor que aquele apresentado por indivíduos não infectados. As médias dos números de células enterogliais S-100^+ nos dois grupos de pacientes chagásicos, portadores e não portadores de megacólon, são semelhantes (Anexo 1).

7. Pacientes chagásicos não portadores de megacólon apresentam um aumento no número de células enterogliais $GFAP^+$, o que não é observado em pacientes portadores de megacólon (Anexo 1).

8. A avaliação da expressão de marcadores neuroquímicos nos plexos nervosos do cólon de pacientes chagásicos portadores de megacólon revelou um aumento da freqüência relativa de neurônios excitatórios Substância P^+ e uma diminuição de neurônios motores inibitórios VIP^+ ou NOS^+ (Anexo 3).

DISCUSSÃO

Dentre os componentes etiológicos do megacólon chagásico, um deles é comprovadamente de natureza imunológica. Estudos anteriores descreveram a presença de ganglionite e peri-ganglionite no cólon de pacientes chagásicos portadores de megacólon, sugerindo a participação de células do sistema imune no desenvolvimento de lesões de estruturas do SNE (Adad *et al.*, 2001; Corbett *et al.*, 2001). Neste estudo apresentamos um estudo descritivo dos focos inflamatórios, bem como de neurônios e células da glia do SNE, visando contribuir para a compreensão da patologia do megacólon chagásico. A partir dos quadros encontrados em pacientes infectados, portadores e não portadores de megacólon, ousamos realizar algumas inferências sobre os possíveis mecanismos envolvidos no desenvolvimento dessa patologia.

Nos pacientes portadores e não portadores de megacólon, os focos inflamatórios, constituídos principalmente de leucócitos mononucleares, foram observados ao longo de todas as túnicas, concentrando-se nas regiões dos plexos nervosos. Dentre as células inflamatórias analisadas, algumas possuem potencial citotóxico bem reconhecido, como eosinófilos, macrófagos, linfócitos T citotóxicos e células *Natural Killer*. Estas foram observadas em focos ou esparsas, nas diversas camadas do cólon. Interessantemente, observamos que o megacólon chagásico está associado com uma alta concentração dessas células e com uma baixa densidade de filetes nervosos PGP 9.5^{+}, o que reforça a hipótese da participação das mesmas (macrófagos, eosinófilos, linfócitos citotóxicos TIA-1^{+}, e células *Natural Killer*) no processo de desnervação induzido pela infecção por *T. cruzi*.

É importante ressaltar que, ao nosso conhecimento, esse é o primeiro estudo a evidenciar a presença de linfócitos TIA-1^{+} nas regiões dos plexos nervosos do cólon de pacientes chagásicos, sugerindo a participação do mecanismo de citotoxicidade mediado por linfócitos T nas lesões teciduais da fase crônica. A confirmação da presença destas células no cólon sustenta a hipótese levantada por d'Avila Reis *et al.* (1993; 2001) de que o mecanismo de citotoxicidade mediado por células T seja um dos mecanismos desencadeados de uma forma genérica pela infecção por *T. cruzi*.

O antígeno TIA-1 confere aos linfócitos o potencial de induzir apoptose na célula alvo (Asano *et al.*, 2005; Michalopoulos *et al.*, 2004; Sato-Kawamura *et al.*, 2003). Além disso, os linfócitos T citotóxicos estocam em seus grânulos outras glicoproteínas importantes em

mecanismos de citotoxicidade como perforinas e granzimas. Enquanto as perforinas são capazes de se polimerizar na membrana plasmática de células alvo formando poros, as granzimas degradam o DNA das mesmas. Neste estudo demonstramos ainda, no cólon de pacientes chagásicos, a presença de células *Natural Killer*, que também estocam granzimas e proteases (Hasenkamp *et al.*, 2006). A presença de células *Natural Killer* em lesões de pacientes chagásicos já havia sido anteriormente demonstrada no cólon de portadores de megacólon (Corbett *et al.*, 2001) e no coração de indivíduos chagásicos cardiopatas (Reis *et al.*, 1993).

É importante ressaltar ainda que, pacientes não portadores de megacólon também apresentaram células com potencial citotóxico, embora em concentrações inferiores àquelas observadas no cólon de indivíduos com megacólon. Além disso, de acordo com a análise da densidade de filetes nervosos PGP 9.5^{+}, esse grupo apresentou um nível de desnervação intermediário, o que sugere que a destruição de componentes do SNE na doença de Chagas é um processo contínuo que se inicia na fase aguda e persiste até a fase crônica sendo, pelo menos em parte, dependente de mecanismos imunológicos. Essa hipótese está de acordo com os trabalhos realizados por Adad (Adad *et al.*, 1991; Adad *et al.*, 2001), os quais demonstraram no esôfago e cólon de pacientes portadores da forma digestiva, uma relação direta entre severidade do processo inflamatório, intensidade de desnervação do órgão e grau de evolução da doença.

A participação de macrófagos no processo de lesão de componentes do SNA já havia sido anteriormente sugerida a partir de análises de coração de ratos infectados com *T. cruzi* (Carvalho *et al.*, 2006; Melo & Machado, 1998; Melo & Machado, 2001). Os macrófagos são células capazes de associação a sítios inflamatórios, promovendo a exacerbação de mecanismos de lesão celular. Essas células secretam citocinas como TNF-α , IL-1β e IL-6, as quais possuem a capacidade de induzir processos de citotoxicidade mediados por outras células do sistema imune. Além disso, macrófagos por si só podem lesar parasitas ou células do próprio hospedeiro, devido a sua capacidade de produzir, quando ativados, substâncias citotóxicas, como óxido nítrico e radicais livres (Daryani *et al.*, 2003).

Demonstramos ainda neste estudo o aumento da concentração de eosinófilos no cólon de pacientes chagásicos, principalmente naqueles portadores de megacólon. Cardoso *et al.* (2006), em relatos anteriores sobre estudos funcionais de células sanguíneas de pacientes com a forma cardio-digestiva da doença Chagas, demonstraram que a principal fonte de citocinas (IFN-γ, TNF-α, IL-12, IL-4, IL-5 e IL-10) nesses indivíduos é o eosinófilo. Interessantemente, neste

estudo aqui apresentado, todos os pacientes apresentavam também cardiopatia chagásica, o que nos incita a especular sobre a participação dos eosinófilos nos processos inflamatórios crônicos, especificamente da forma cardio-digestiva. Em relatos de estudos anteriores, Molina e Kierszenbaum (1989) observaram no coração de pacientes chagásicos portadores de cardiopatia, depósitos de uma neurotoxina derivada de eosinófilos, bem como a presença de eosinófilos ativados (Molina & Kierszenbaum, 1989a). Milei *et al.* (1991) também demonstraram no coração de pacientes chagásicos cardiopatas a presença de eosinófilos, em 5% dos casos analisados. No entanto, nenhum desses estudos citados menciona a ocorrência concomitante da forma digestiva nos pacientes estudados.

Os eosinófilos possuem grânulos intra-citoplasmáticos ricos em proteínas básicas e outras enzimas, dentre elas a proteína básica principal (MBP), proteína eosinofílica catiônica (ECP), peroxidase eosinofílica (EPO) e a neurotoxina derivada de eosinófilos (EDN) (Silberstein *et al.*, 1989; Spry, 1989).Além de expressar uma série de moléculas co-estimulatórias (CD40, CD28, CD86, B7.1 e B7.2) (Ohkawara *et al.*, 1996; Woerly *et al.*, 1999), os eosinófilos secretam citocinas pró- e anti-inflamatórias, como as IFN-γ, TNF-α, IL-12, IL-4, IL-5 e L-10 (Kita, 1996; Lucey *et al.*, 1989). No intestino, eosinófilos são encontrados principalmente associados a processos inflamatórios agudos e crônicos, e seu papel na manutenção da fisiologia intestinal é bem conhecido (Weller, 1997; Weller & Lim, 1997). Então como podemos constatar pela breve descrição da biologia do eosinófilo, ele é uma célula de várias facetas, podendo ativar ou inibir processos inflamatórios, promover lesão de parasitas e células e ainda participar da fisiologia intestinal. No megacólon chagásico, o papel real dos eosinófilos só poderá ser determinado através da análise do seu estado funcional, não apenas em lesões de pacientes chagásicos, mas também em modelos experimentais.

Outra observação relevante, apesar de não ser inédita, foi a mastocitose no cólon dos pacientes chagásicos portadores de megacólon. Os mastócitos são células efetoras multifuncionais do sistema imune e possuem um papel importante na fisiologia e defesa contra infecções parasitárias (Skaper *et al.*, 2001). Acreditamos que a mastocitose observada no cólon de indivíduos chagásicos portadores de megacólon seja um fator importante na exacerbação e manutenção do processo inflamatório neste grupo de pacientes. Além de promover o aumento da permeabilidade vascular, o mastócito estoca, em seus grânulos, substâncias inflamatórias como por exemplo prostaglandinas, TNF-α e IL-6, as quais são capazes de estimular a expressão de

moléculas de adesão pelo endotélio promovendo a migração de leucócitos (Bendixsen *et al.*, 1995; Bischoff *et al.*, 1999a; Bischoff *et al.*, 1999b; Lorentz *et al.*, 2000).

O aumento do número de mastócitos no megacólon chagásico foi também relatado na literatura científica por outros autores (Pinheiro *et al.*, 2003; Tafuri *et al.*, 1971). Estes autores demonstraram que a mastocitose nos pacientes portadores da forma digestiva é também acompanhada de um processo de fibrose. Neste estudo, nós não apenas confirmamos essas observações, mas também, através de uma análise morfométrica, demonstramos que a área de fibrose se co-relaciona com a concentração de mastócitos, eosinófilos e macrófagos. Sabe-se que certas citocinas, como TNF-α , TGF-β1, IL-1β e IL-4, produzidas em grandes quantidades em processos inflamatórios, principalmente por células do sistema imune, possuem capacidade de induzir diretamente o processo de fibrinogênese ao ativar miofibroblastos (Porter *et al.*, 2004). É possível que a exacerbação do processo inflamatório em pacientes chagásicos portadores de megacólon leve a um aumento na produção e síntese destas citocinas, o que se refletiria no aumento da área de fibrose nas camadas musculares desses pacientes. Além disso, é importante considerar o papel dos macrófagos no processo de remodelação, enquanto célula produtora de colagenases (Otte *et al.*, 2003). Acreditamos que futuros estudos visando à caracterização dos mediadores envolvidos no recrutamento dessas células inflamatórias possam ajudar a definir métodos terapêuticos no sentido de evitar ou mesmo amenizar o desenvolvimento do megacólon chagásico.

Uma outra abordagem de estudo aqui utilizada constou da quantificação de sub-populações de células enterogliais, através da qual demonstramos, nas regiões de plexo nervoso do cólon de ambos os grupos de pacientes chagásicos, uma diminuição significativa dessa população celular S-100^{+}. Da mata *et al.* (2000), ao avaliar o SNC em ratos infectados por *T. cruz*, demonstraram uma preferência do parasita pelos astrócitos. A partir dessas observações, os autores sugerem ser as alterações neuronais encontradas, uma conseqüência da destruição da glia e do processo inflamatório desencadeado pelo parasitismo celular. É possível que no SNE humano, ocorra também uma preferência do parasita pela célula enteroglial, mas isso seria um fenômeno observável somente na fase aguda da doença, quando o parasitismo ainda é grande. Apesar de ser esse um estudo da fase crônica, os dados obtidos nos incitam a especular sobre a validade dessa hipótese para a infecção humana. Demonstramos a semelhança estatística entre as médias dos números de células enterogliais S-100^{+} nos dois grupos de pacientes analisados,

portadores e não portadores de megacólon, o que sugere que a morte da glia entérica deva acontecer principalmente na fase aguda da infecção, quando o parasitismo ainda é grande, ou pelo menos precocemente na fase crônica da doença, não guardando relação direta com o desenvolvimento do megacólon chagásico.

As células enterogliais foram também analisadas quanto à expressão de GFAP, uma proteína estrutural, não constitutiva, da classe dos filamentos intermediários. Foi interessante observar que, enquanto nos pacientes portadores de megacólon o número de células enterogliais $GFAP^+$ não está alterado em relação aos pacientes não infectados, no grupo de pacientes chagásicos não portadores de mega observa-se um aumento do número dessas células. Sendo um constituinte dos filamentos intermediários, uma das funções atribuída à glicoproteína GFAP é a de contribuir para aumentar a coesão entre as células da glia, criando assim uma barreira de proteção para os corpos neuronais (Buniatian *et al.*, 2002). Assim, o aumento da expressão de GFAP nos pacientes não portadores de megacólon, pode representar uma tentativa de proteção de componentes do SNE contra fatores lesivos inerentes ao processo inflamatório ou mesmo ao próprio parasita. Partindo deste pressuposto, o desenvolvimento do megacólon poderia ser explicado, pelo menos em parte, pela incapacidade daqueles indivíduos em aumentar a expressão de GFAP em suas células enterogliais.

Na doença inflamatória intestinal, a glia entérica aparentemente realiza um papel central no controle da inflamação (Geboes *et al.* 1992; Ruhl & Collins, 1995). Bush *et al.* (1998) ao depletar células $GFAP^+$ do trato gastrintestinal de camundongos adultos observaram que em apenas duas semanas todos os animais morreram de jejunoileíte fulminante. Estudos sobre enterocolite necrosante (Cornet *et al.*, 2001) demonstraram que intensos processos inflamatórios são acompanhados por uma severa diminuição do número de células da glia $GFAP^+$. Também na doença de Chagas é possível que as células $GFAP^+$ tenham um papel relevante no controle da inflamação, pois de acordo com os dados aqui apresentados, elas são mais concentradas justamente no grupo que apresenta um processo inflamatório mais brando, ou seja, nos pacientes chagásicos não portadores de megacólon.

Segundo Koberle (1968), para o desenvolvimento do megacólon ou do megaesôfago é preciso que haja uma destruição neuronal acima de 50% e de 80%, respectivamente. No entanto, de acordo com estudos anteriores de pacientes chagásicos portadores e não portadores de megaesôfago, alguns pacientes chagásicos não portadores de megaesôfago apresentam um

processo de desnervação bem elevado, podendo chegar a 85%, enquanto pacientes com a doença digestiva mostram uma redução neuronal média de 60%.(da Silveira *et al.*, 2005b). A partir desses dados ousamos contra-argumentar com Koberle (1968) e sugerimos que o desenvolvimento do mega não pode ser justificado somente por uma destruição quantitativa de neurônios, mas também por uma eliminação seletiva de certas classes neuronais. De fato, este estudo veio, pelo menos em parte, corroborar com essa hipótese. Demonstramos que no megacólon chagásico acontece uma destruição preferencial de neurônios motores inibitórios VIP^+ e $nNOS^+$, e um aumento na freqüência de neurônios substância P^+.

Níveis elevados de substância P em neurônios entéricos já foram detectados no cólon de pacientes portadores de colite ulcerativa (Bernstein *et al.*, 1993; Koch *et al.*, 1987) e doença inflamatória intestinal (Mantyh *et al.*, 1994), estando diretamente co-relacionados com a atividade de tais patologias. Acreditamos que na doença de Chagas o aumento relativo da expressão de substância P no cólon de pacientes portadores de megacólon represente um aumento da expressão deste neuropeptídeo por neurônios que anteriormente não o expressavam. Por outro lado, também não excluímos a hipótese que por se tratar de uma freqüência relativa, esse "aumento" da expressão de substância P seja na verdade uma menor destruição neste grupo neuronal. A substância P age sobre o sistema imune principalmente em macrófagos, linfócitos T e B, induzindo a síntese e secreção de várias citocinas pró-inflamatórias. Dentre essas, podemos destacar o TNF-α, IL-1β, IL-6 e IL-2 (Holzer & Holzer Petsche, 1997). Ela pode ainda ativar mastócitos induzindo a liberação das diversas substâncias inflamatórias contidas nos seus grânulos (Raithel *et al.*, 1999). Por vias indiretas, a substância P induz a proliferação de linfócitos e ativação de células *Natural Killer*, aumenta a expressão de moléculas de adesão por células endoteliais e estimula a migração de leucócitos para sítios inflamatórios (Holzer, 1998; Laurenzi *et al.*, 1990). Então é possível que o aumento da expressão de substância P por neurônios entéricos seja uma das causas da exacerbação do processo inflamatório observado em pacientes chagásicos portadores de megacólon. Acreditamos que substâncias capazes de antagonizar a Substância P ou de bloquear os seus receptores (NK1, NK2, NK3) possam desempenhar papel terapêutico importante no controle da imunopatologia do megacólon chagásico.

Como já mencionado anteriormente, as freqüências dos neurônios motores inibitórios $nNOS^+$ ou VIP^+ estão diminuídas nos pacientes chagásicos portadores de megacólon quando comparadas com aquelas apresentadas por indivíduos controle não infectados. Julgamos que a

diminuição desses neurônios represente um mecanismo crucial para a implantação do megacólon chagásico. No cólon, os neurônios que expressam nNOS e VIP são responsáveis pelo relaxamento muscular observado durante a peristalse. A distensão local dentro do lúmen do cólon induz um reflexo neural e em conseqüência uma contração muscular proximal e um relaxamento distal (Sanders *et al.*, 1992). Assim, a depleção de neurônios motores inibitórios no SNE pode ter como conseqüência uma diminuição do trânsito intestinal. Alterações similares foram observadas na doença de Hirschsprung, caracterizada por uma profunda diminuição em corpos neuronais nos plexos submucoso e mientérico do cólon (Guo *et al.*, 1997; Kusafuka & Puri, 1997).

Trabalhos anteriores mostram que o relaxamento da porção interna do esfíncter anal pode ser estimulado pelo uso tópico de trinitrato de gliceril, uma substância doadora de óxido nítrico, com resultados relativamente bons (Loder *et al.*, 1994; Lund & Scholefield, 1997). No futuro, acreditamos que procedimentos que induzam à elevação dos níveis de NO e VIP no intestino, tais como transfecção viral ou drogas específicas, possam ser utilizados para impedir ou minimizar o desenvolvimento do megacólon chagásico.

CONCLUSÕES

As observações obtidas neste trabalho nos permitem apresentar as seguintes suposições a respeito do desenvolvimento do megacólon chagásico:

- As lesões teciduais que ocorrem na fase crônica do megacólon chagásico são possivelmente resultantes de mecanismos de citotoxicidade diversos, dentre eles aqueles mediados por linfócitos T TIA-1^+, células *Natural Killer*, macrófagos e eosinófilos.

- Os macrófagos, os eosinófilos e os mastócitos possivelmente participam do processo da fibrose observada nas camadas musculares do cólon de pacientes chagásicos portadores de megacólon.

- O desenvolvimento do megacólon chagásico parece ser explicado não apenas pela taxa de morte neuronal, mas também pela freqüência de destruição de cada classe de neurônio. A destruição seletiva de neurônios motores inibitórios $nNOS^+$ e VIP^+ e o aumento da freqüência de neurônios substância P^+ parecem propiciar o desenvolvimento dessa patologia.

- A destruição de células enterogliais na infecção chagásica parece acontecer precocemente e não se co-relaciona com desenvolvimento do megacólon.

- É possível que as células enterogliais $GFAP^+$ participem da modulação do processo inflamatório e, conseqüentemente, da proteção neuronal e do controle do desenvolvimento do megacólon chagásico.

Os dados aqui apresentados servirão como referência para trabalhos posteriores, em outros modelos experimentais, nos quais possam ser comprovadas nossas especulações a respeito dos mecanismos de desenvolvimento do megacólon chagásico.

PERSPECTIVAS

No modelo humano de megacólon chagásico:

1. Analisar a produção de citocinas e fatores neurotróficos por células inflamatórias e neurônios.

2. Estudar o processo regenerativo de filetes nervosos através de dupla marcação para as diversas classes neuroquímicas e para marcadores de regeneração, como por exemplo, o GAP-43.

3. Investigar a presença de células reguladoras através de marcação para FOXp3.

No modelo experimental:

1. Avaliar o papel da substância P na inflamação intestinal induzida pela infecção por *T. cruzi*, através de estudos de bloqueio de seus receptores.

2. Avaliar o papel da GFAP na modulação do processo inflamatório intestinal induzido pela infecção por *T. cruzi*, através da utilização de camundongos geneticamente modificados.

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ANEXO 1

Ms. Ref. No.: YHUPA-D-06-00531R1
Title: MEGACOLON IN CHAGAS' DISEASE: A STUDY OF INFLAMMATORY CELLS, ENTERIC NERVES AND GLIAL CELLS
Human Pathology

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MEGACOLON IN CHAGAS' DISEASE:

A STUDY OF INFLAMMATORY CELLS, ENTERIC NERVES AND GLIAL CELLS

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Keywords: chagasic megacolon, inflammatory cells, enteric nervous system, glial cells, enteric nerves

ABSTRACT

Following acute infestation with the Chagas' disease parasite, *T. cruzi*, some patients who are serologically positive develop chronic megacolon and megaesophagus, while others are symptom free. Chagas' disease with gastrointestinal involvement involves an inflammatory invasion of the enteric plexuses and degeneration of enteric neurons. It is known that glial cells can be involved in enteric inflammatory responses. The aims were to determine the nature of any difference in lymphocytic invasion, enteric neurons, and enteric glial cells in sero-positive individuals with and without megacolon. We have compared colonic tissue from serologically positive individuals with and without symptoms and from sero-negative controls. Subjects with megacolon had significantly more CD-57 natural killer cells and TIA-1 cytotoxic lymphocytes within enteric ganglia, but numbers of CD-3 and CD-20 immunoreactive cells were not significantly elevated. The innervation of the muscle was substantially reduced, to about 20% in megacolon, but asymptomatic sero-positive subjects were not different to sero-negative controls. Glial cell loss occurred equally in symptomatic and unaffected sero-positive subjects, although the proportion with glial fibrillary acidic protein was greater in sero-positive, non-symptomatic, subjects. Development of megacolon following acute infection with *T. cruzi* is associated with maintained invasion of enteric ganglia with cytotoxic T cells and loss of muscle innervation, but changes in glial cell numbers is not associated with progression of enteric neuropathy.

INTRODUCTION

Chagas' disease, caused by the protozoan parasite *Trypanosome cruzi* (*T. cruzi*), is an endemic cause of morbidity and mortality in areas of Central and South America [1]. There are many immigrants to the United States and other countries from these areas who are chronically infected. A current estimate suggests that there are approximately 100,000 infected individuals in the United States [2].

Acute Chagas' disease is characterized by fever and myocarditis related to intracellular parasitism. Usually these symptoms subside spontaneously. Most patients then remain serologically positive, but asymptomatic, for the rest of their lives. However, some patients, after a prolonged asymptomatic period (20–30 years), can exhibit chronic Chagas' disease, which is usually manifested by cardiomyopathy and gross enlargement of the tubular structures of the gastrointestinal system, particularly megaesophagus and megacolon [3,4].

In megacolon, motility disturbances are associated with the enlargement of the colon and constipation. The rectum and the sigmoid colon are the most compromised segments [5], which exhibit striking luminal enlargement and muscular hypertrophy. Inflammatory lesions in the enteric nervous system (ENS) are associated with a substantial reduction in the number of neurons. This loss of neurons has been thought to underlie the clinical findings in megasyndromes [6].

Information is still scarce regarding changes in the ENS after *T. cruzi* infection and the potential role of immune cells in the development of chagasic megacolon. It is possible that there is a chronic inflammation of enteric ganglia in the infected subjects who develop late gastrointestinal symptoms, but this possibility has not been tested. Also, because of the reaction of enteric glia when the enteric ganglia are inflamed [7], these cells are a potential intermediate in

the effects on enteric neurons. We have therefore compared, quantitatively and qualitatively, the presence of inflammatory cells in enteric ganglia, changes in enteric innervation of the muscle and enteric glial cell numbers in chagasic patients with and without megacolon.

MATERIAL AND METHODS

Patients and Samples

Samples of full thickness colon wall tissue were obtained from 8 chagasic patients with megacolon, 8 chagasic patients without megacolon and 10 control individuals submitted to necropsy or surgical procedures at Faculdade de Medicina do Triângulo Mineiro (Uberaba, Minas Gerais, Brazil). Patients did not receive any parasite-specific treatment. Informed consent was obtained from the patient or family members prior to tissue procurement. This work was approved by UFMG Research Ethics Committee.

Serological tests indicative of Chagas' disease (complement fixation, hemagglutination, and immunofluorescence tests) were positive in all patients studied. All of the patients had left the endemic area more than 20 years before the tissue collection, and during that time, patients without megacolon did not present any symptom related to digestive disease. All patients originated from Uberaba, MG, Brazil, where the natural transmission of Chagas' disease was interrupted more than 20 years ago, and they had never received blood transfusions. The patients with and without megacolon had mean ages of 57 ± 10 years, and 55 ± 14 years respectively. The presence of megacolon was established based in clinical data reporting colon obstruction, and from radiological studies. Manometric studies of megacolon demonstrated decreased peristalsis

and incomplete relaxation of the anal sphincter. These abnormalities precede clinical symptoms and dilatation seen by radiographic studies.

The control group was composed of non-infected individuals, as indicated by negative serology specific for Chagas' disease. Non-infected individuals were also from the state of Minas Gerais and had a mean age of 54 ± 20 years.

Histology and peroxidase immunohistochemistry

Tissue samples were collected from the recto-sigmoid region. Each specimen was fixed in 4% neutral buffered formaldehyde solution and embedded in paraffin for immunohistochemistry studies. Sections of 7µm were deparaffinized using xylene, and rehydrated through graded alcohols. Some sections were then stained for standard histology, using haematoxylin and eosin (H&E) while others were prepared for immunohistochemistry. For immunohistochemistry, endogenous peroxidase was inhibited by incubation with 1% hydrogen peroxide and 30% absolute methanol for 30 min. The slides were then incubated with 2% normal swine serum (NSS) (Sigma, USA) in phosphate buffered saline (PBS) for 15 min and subsequently with the following monoclonal antibodies: anti-PGP 9.5 (Santa Cruz Biotechnology, USA, 1:1000) for neural fibers; anti-S-100 and anti-GFAP (Santa Cruz Biotechnology, USA, 1:2000 / DAKO, 1:500, USA) for glial cells; anti-CD3 for T lymphocytes; anti-CD20 (DAKO, USA, 1:100) for B lymphocytes; anti-CD57 (Santa Cruz Biotechnology, clone sc-6261, 1:200) for Natural Killer (NK) cells, and anti-TIA-1 (Santa Cruz Biotechnology, clone sc-1751, 1:200). Following this, the tissue sections were incubated with peroxidase-conjugated rabbit anti-mouse antibodies (DAKO) for 45 min, and peroxidase activity was detected by incubation with 3,3'-diaminobenzidine (Sigma) and hydrogen peroxide for 10 min. Slides were counterstained with Gill's haematoxylin

(Sigma), dehydrated in graded alcohols, and mounted in synthetic mounting media. Negative control slides without primary antibody were performed for each case.

Cell quantification and morphometric studies

Enumeration of glial cells was performed in the submucosal and myenteric plexuses, while the inflammatory cells counting were performed in all colon layers (nervous plexuses and muscle layers), by counting 20 randomly-selected fields (total area of 1066μm^2) on a single slide per patient. Morphometric studies of neuronal fibres were performed by image analysis (Kontron KS300 v. 2.0), by measuring PGP 9.5 immunoreactivity (IR) areas on 20 randomly-selected fields (total area of 1066μm^2) in the muscle layers on a single slide per patient.

Statistics

Statistical analysis among the different groups was analysed by one-way ANOVA. Differences were considered statistically significant at $P<0.05$.

RESULTS

Analysis of inflammatory cells

H&E sections of colon from patients with megacolon demonstrated that myenteric and submucosal plexuses were damaged, with necrosis of ganglion cells, inflammatory responses and fibrosis. Accumulations of immune cells extended into the outer layers of the mucosa (Figure 1).

Mild infiltration of mononuclear cells around degenerated ganglion cells was observed in all cases. All these findings were also observed in colon from chagasic patients without megacolon although, in this group, normal ganglion cells, without signs of degeneration, were frequently observed. The control group, which was composed of non-infected individuals, did not show any significant inflammatory process (Figure 1). In particular, lymphocytes were rare within or close to enteric ganglia of control tissues.

Table 1 and Figure 2 show the results of the characterization of inflammatory cells in the colon plexuses and muscle layers of chagasic patients. We demonstrated, in patients with and without megacolon, the presence of CD3-IR T lymphocytes, CD20-IR B lymphocyte, CD57-IR NK cells, and TIA-1-IR cytotoxic T lymphocytes. In both groups, with and without megacolon, there was a clear predominance of CD3-IR T lymphocytes in the myenteric plexus and muscle layers, and CD20-IR B lymphocytes in the submucosal plexus. Quantitative comparative analysis of the inflammatory cells, in subjects with and without megacolon, demonstrated that there were greater numbers of both CD57-IR NK cells and TIA-1-IR cytotoxic T lymphocytes in the enteric plexuses of affected subjects.

Assessment of innervation

Assessment of innervation was performed by using an anti-PGP 9.5 monoclonal antibody, that has high specificity for neurons and nerve fibres [1-3]. The distribution of PGP 9.5 immunoreactivity was similar in all groups analysed, being observed in every intestinal layer.

Representative aspects of PGP 9.5-IR nerve fibers in each group are shown in Figure 3. Nerve fibers observed in chagasic groups were thinner than those in samples from the control group (Figure 3). This is interpreted as a sign of loss of axons from nerve fibre bundles in the

muscle. Computerized morphometric analysis of PGP 9.5-IR in the muscle layers revealed reduction of the integrated areas of nerve fibers in patients with megacolon, when compared to the other two groups (Figure 4). Although the sizes of PGP 9.5-IR nerve fibers in patients without megacolon were smaller than the normal pattern (Figure 3), there was not any significant difference in fibre distribution between these two groups (Figure 4).

Quantification of glial cells

Analyses of glial cells were performed by immunoreaction with two different antibodies: anti-S-100 (pan-glial marker) and anti-GFAP (glial sub-population marker) [1,2]. It was observed that the antibody against S-100 protein exclusively labels glial cells, with no staining in the neuronal cell bodies (Figure 5a). Qualitative observations revealed weaker S-100 immunoreactivity in nerve fibers of patients with megacolon, and morphometric analyses demonstrated significant and approximately equal loss of S-100-IR glial cells in both populations of chagasic patients (Figure 6).

We also analyzed the expression of GFAP by enteric glial cells in the colon. The antibody against GFAP stained glial cells exclusively, with no staining of neuronal cell bodies and processes (Figure 5b). Quantification of GFAP-IR cells demonstrated that chagasic patients without megacolon have an increased number of GFAP-IR cells compared with other groups (Figure 7). No statistical difference on the number of GFAP-IR glial cells was observed between chagasic patients with megacolon and non-infected individuals.

DISCUSSION

Neuronal destruction has been considered the hallmark of chagasic megacolon. In this study, we confirm this suggestion by demonstrating that patients with megacolon have decreased density of PGP 9.5-IR neuronal fibers within the external muscle when compared to control group or even to chagasic patients without megacolon. Sero-positive subjects without megacolon did not show any statistically difference when compared to the control group. Previous studies have shown a small degree of neuronal degeneration in asymptomatic sero-positive subjects [6].

The cause of neuronal destruction in Chagas' disease has been debated in the literature [6, 13, 14]. In the acute phase, when *T. cruzi* is present in high concentration in the tissue, the parasite might be responsible for the neuronal destruction [13, 15, 16]. In contrast, in the chronic phase the parasite load is very low in the chagasic lesions, and parasite DNA has been detected mainly by the polymerase chain reaction [17, 18]. Thus, it has been suggested that chronic neuronal destruction might be a consequence of the immune response that follows infection. Although ganglionitis and myositis have been described in the colon of chagasic patients, the phenotypes of inflammatory cells associated with the lesions have not been extensively studied. Here we show that the inflammatory infiltration, in both groups of sero-positive subjects, is composed mainly of CD3-IR T lymphocytes and CD20-IR B-lymphocytes, which is in agreement with data reported by Corbett *et al.* who analysed the colons of patients with megacolon [19]. We further show, in colon lesions, the presence of NK cells and TIA-1 cytotoxic cells is greater in patients with megacolon, which supports the participation of the immune response in the neuronal loss that occurs many years after the acute infective episode. The presence of TIA-1-IR cytotoxic lymphocytes and NK cells has been previously demonstrated in the heart [20] and esophagus [21] of patients with chagasic cardiomyopathy and megaesophagus,

respectively, pointing to a common mechanism of tissue damage in the *T. cruzi* induced pathology.

We also demonstrate here the loss of another important component of the ENS, the S-100-IR glial cells, in the colon of chagasic patients. Loss of enteric glial cells has been reported in other intestinal diseases [22, 23]. Recent evidence indicates that enteric glial cells play an active role in the control of gut physiology and pathophysiology [7], and that they are activated specifically and participate actively in the course of intestinal inflammation [12]. In two transgenic mouse models, fulminating tissue inflammation and necrosis ensue when the enteric glial cells network is disrupted [22, 24]. In view of those data, we could speculate that the loss of enteric glial cells in Chagas' disease might induce loss of colon homeostasis and contribute to the development of chronic megacolon.

Two classes of glial cells can be normally distinguished in the enteric nervous system, namely the GFAP-IR group expressing high levels of GFAP, and the GFAP non-reactive group. Von Boyen et al infer that glial cells increase the expression of GFAP when they are in contact with pro-inflammatory cytokines [12]. Increased GFAP expression has been observed in some inflammatory diseases of the gut such as ulcerative colitis and Crohn's disease. In Chagas' disease, it was interesting to observe that, despite the overall loss of enteric glial cells, the patients presented increasing percentages of the GFAP-IR population. Being a constituent of the intermediate filaments, one of the functions of GFAP is possibly to increase the cohesion between the glial cells [25]. Considering these observations, it is tempting to speculate that the increased expression of GFAP-IR glial cells in chagasic patients creates a barrier of protection for the neuronal cell bodies, and represents thus an attempt to protect ENS neurons against inherent harmful factors of inflammatory processes or against parasite infection. This hypothesis is in

agreement with our data demonstrating that increased percentage of GFAP-IR cells is observed mainly in patients without megacolon.

All of the patients analysed in this study had left the endemic area more than 20 years ago, but not all of them presented with megacolon. In the past, by analysing the esophagus of chagasic patients with and without megaesophagus, we observed that loss of neural tissue in the organ is observed not only in patients with megaesophagus, but also, to a lesser extent, in asymptomatic chagasic individuals [16, 26]. We believe that qualitative comparative studies on the types of neurons affected in these populations will help in understanding the pathogenesis of megabowel in Chagas' disease. It is possible that in patients with megaesophagus and megacolon some categories of neurons and some particular enteric neurotransmitter systems could be affected more than others.

Finally, we believe that the inflammatory process and glial cell alterations described in the colon of chagasic patients might disrupt the enteric nervous system and contribute to development of the pathology, including the degenerative process in the ganglia. Ongoing studies on the characterization of signalling molecules that mediate the exchange of information between these cells [27], including cytokines, neurotransmitters and neurotrophic factors, may help in understanding the pathogenesis of chagasic megacolon.

ACKNOWLEDGEMENTS

This work was supported by funds from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Ministério da Educação, Brazil and the National Health and Medical Research Council of Australia (grant 400020)

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Quantification of inflammatory cells in the colon of chagasic patients with and without megacolon (number of cells / mm^2)								
Cells	**Chagasic patients without megacolon**				**Chagasic patients with megacolon**			
	CD3-IR lymphocyte	CD20-IR lymphocyte	CD57-IR Natural Killer cells	TIA-1-IR cytotoxic lymphocyte	CD3-IR lymphocyte	CD20-IR lymphocyte	CD57-IR Natural Killer cells	TIA-1-IR cytotoxic lymphocyte
Submucosal plexus	28 ± 11	43 ± 13	8 ± 2*	7 ± 2*	33 ± 13	52 ± 14	14 ± 3	23 ± 4
Myenteric plexus	41 ± 11	4 ± 1	18 ± 3*	11 ± 2*	58 ± 14	6 ± 2	38 ± 6	27 ± 4
Inner muscle layer	10 ± 3	4 ± 1	9 ± 3	3 ± 1	16 ± 3	5 ± 1	12 ± 3	5 ± 2
Outer muscle layer	14 ± 3	5 ± 1	11 ± 3	5 ± 1	19 ± 4	5 ± 1	14 ± 3	6 ± 2

Table 1 – Quantification of CD3-IR lymphocytes, CD20-IR lymphocytes, CD57-IR NK cells and TIA-1-IR cytotoxic T lymphocytes on the nervous plexuses and muscle layers of the colon from chagasic patients with and without megacolon. The values are expressed as mean number of cell number ± S.D. (*) Statistically significant differences observed between the two groups. Total area of 1066μm^2 from each field for all patients was analysed. ($P<0.05$).

Figure 1 – H&E stained sections of the colon from control (a, a') and a chagasic patient with megacolon (b, b'). (a) non-infected individual: there is no evidence of inflammatory process in the muscle layers and myenteric plexus; (a') higher magnification showing the presence of a ganglion with intact neurons (arrow) (b) chagasic patient with megacolon: presence of chronic inflammatory process with intense and nodular mononuclear inflammatory infiltration in muscle layers and myenteric plexus; (b') higher magnification showing the presence of inflammatory cells around a degenerated neuronal ganglion (arrow).

Figure 2 – Aspects of the inflammatory process in the submucosal plexus of colon representative of chagasic patients with megacolon. CD3-IR T lymphocytes (a) and CD20-IR B lymphocytes (b) are observed in the submucosa of the colon from patients with megacolon. Inflammatory infiltrations are commonly founded in those patients. CD57-IR NK cells (c) and TIA-1-IR T lymphocytes (d) are also observed in the colon of those patients, sometimes associated with neurons (black arrow, figure c) or near blood vessels (black arrow, figure d). Calibration: 100 µm.

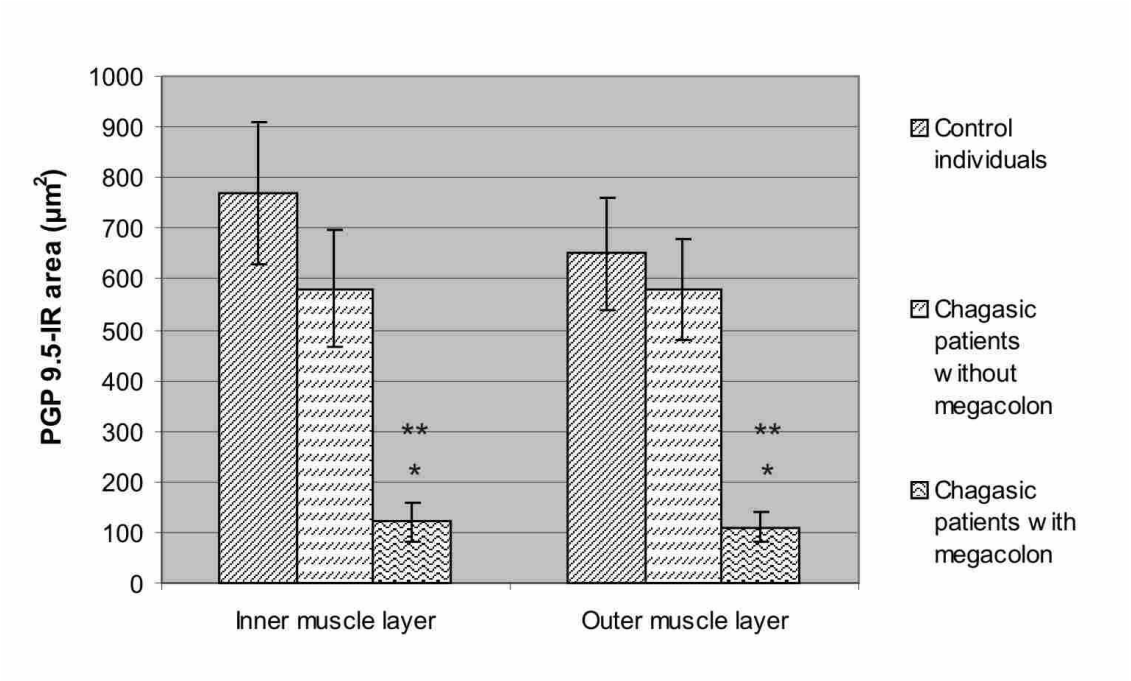

Figure 3 – Morphometric analyses of PGP 9.5-IR nerve fibers in chagasic patients with and without megacolon and control individuals. The values are expressed as means of PGP 9.5-IR areas ± S.D. (*) Statistically significant differences between this group and control individuals. (**) Statistically significant differences between this group and chagasic patients without megacolon group. Total area of 1066µm^2 from each field for all patients was analysed. ($P<0.05$).

Figure 4 - Characterization PGP 9.5-IR nerve fibers in the muscle layers from chagasic patients with and without megacolon and from control individuals. (a) Non-infected individual: nerve fibres can be easily observed in the muscular layer (arrow); (b) chagasic patient without megacolon: nerve fibres are thinner when compared with non-infected individual (arrow); (c) chagasic patient with megacolon: a marked decreased area of PGP 9.5-IR neuronal fibers was observed (arrow). Chagasic patients present a small (b) and advanced (c) neuronal degeneration in the muscle layers.

Figure 5 – Labelling of S-100-IR (a) and GFAP-IR (b) glial cells in the myenteric plexus from chagasic patients with megacolon. Both antibodies showed immunoreactivity only to glial cells and not to neuronal cell bodies (black arrows). Calibration: 50 µm.

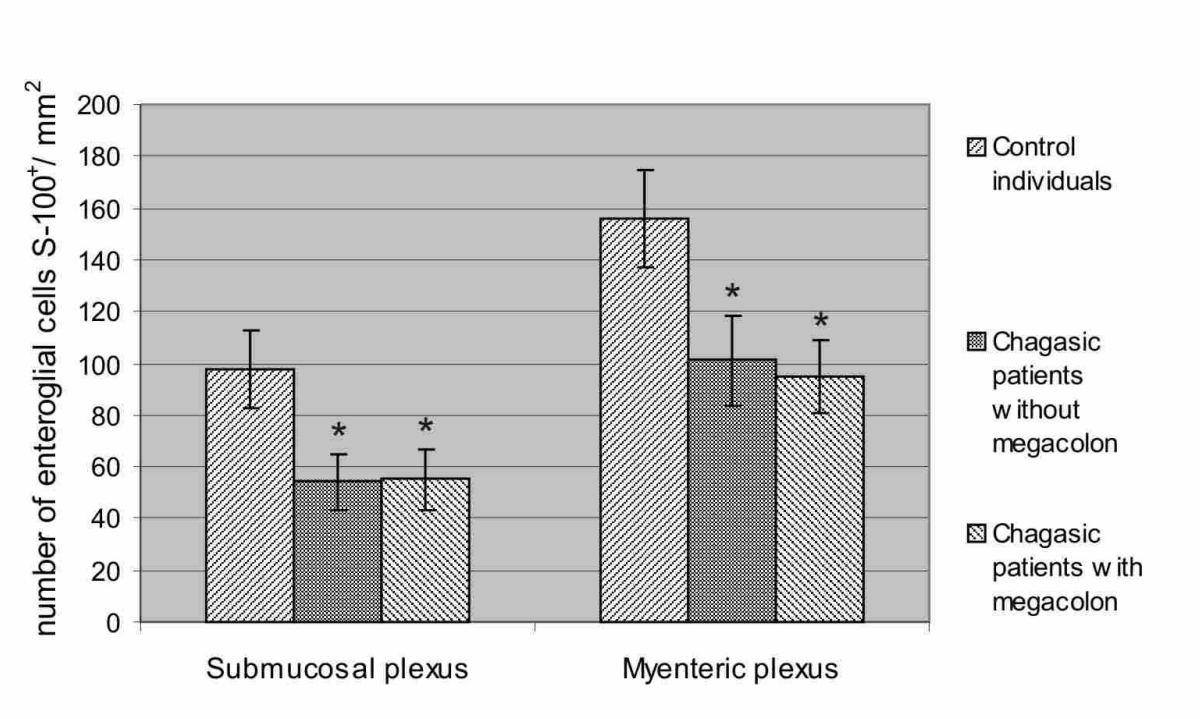

Figure 6 – Quantification of S-100 IR glial cells from chagasic patients with and without megacolon and from control individuals. The values are expressed as means of S-100-IR cell number ± S.D. (*) Statistically significant differences observed between the two groups. Total area of 1066µm^2 from each field for all patients was analysed. ($P<0.05$).

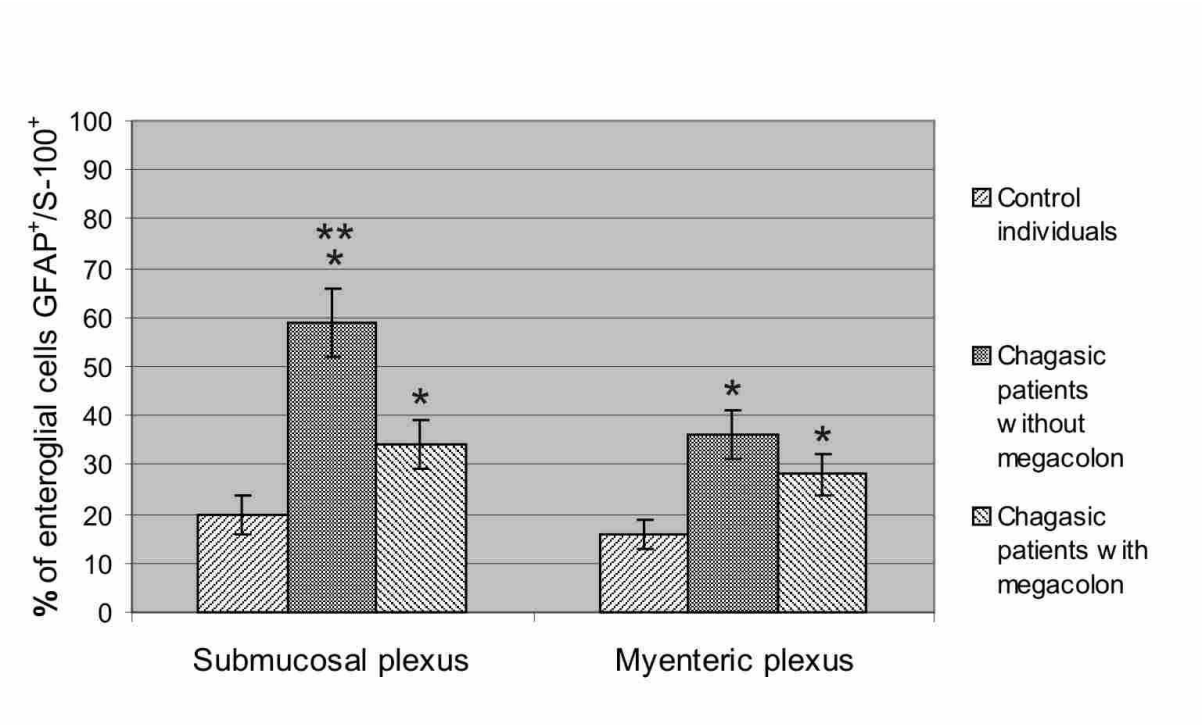

Figure 7 – Ratio of GFAP-IR glial cells in chagasic patients with and without megacolon and non-infected control individuals ± S.D. (*) Statistically significant differences between this group and control individuals. (**) Statistically significant differences between this group and chagasic patients with megacolon group. Total area of 1066 μm^2 from each field for all patients was analysed. ($P<0.05$).

ANEXO 2

27 – Nov – 2006

From: s.phillips@bio.gla.ac.uk
To: alec@icb.ufmg.br

Subject: PARASITOLOGY: Manuscript Accepted MS ID PAR-2006-0266.R1

Dear Dr. Da Silveira

MS ID: PAR-2006-0266.R1

Title: MORPHOMETRIC STUDY OF EOSINOPHILS, MAST CELLS, MACROPHAGES AND FIBROSIS IN THE COLON OF CHRONIC CHAGAS PATIENTS WITH AND WITHOUT MEGACOLON

I am pleased to inform you that your manuscript **has been accepted** for publication in PARASITOLOGY and will now be sent to Press.

Yours sincerely,

Stephen Phillips

Prof. Stephen Phillips

MORPHOMETRIC STUDY OF EOSINOPHILS, MAST CELLS, MACROPHAGES AND FIBROSIS IN THE COLON OF CHRONIC CHAGAS PATIENTS WITH AND WITHOUT MEGACOLON

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Running title: Study of the participation of eosinophils, mast cells, macrophages in the occurrence of fibrosis in the colon of chronic chagas patients with and without megacolon

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SUMMARY

The mechanisms involved in the development of chagasic megacolon are not completely understood. Although autoimmunity may play a role in the pathogenesis of Chagas' disease, recent studies suggest a positive correlation between tissue parasitism, inflammation, and severity of lesions. The aim of this study was to evaluate the presence of inflammatory cells and the occurrence of fibrosis in the colon of chagasic patients with and without megacolon. Samples from 26 patients were randomly selected and paraffin embedded tissue blocks were sectioned and analyzed by histology and immunohistochemistry. It was evaluated the presence of eosinophils, mast cells, and macrophages, as well as the correlation between the concentration of these cells and the area of fibrosis. The analyzes of tissue sections revealed that, the greater concentration the inflammatory cells analyzed, the more extensive area of fibrosis is observed, suggesting the occurrence of a continuous process of cell damage in the chronic phase of chagasic megacolon.

Keywords: chagasic megacolon, eosinophils, mast cells, macrophages, fibrosis

INTRODUCTION

Chagas' disease, caused by the hemoflagellate protozoan *Trypanosoma cruzi* (*T. cruzi*), is one of the few functional gastrointestinal disorders for which a causative agent has been identified. This disease affects 11 million people, in the Latin America and Southeast ranging from the United States-Mexico border thought to Argentina and Chile (Moncayo, 2003). Although control programs directed at decreasing transmission of *T. cruzi* to humans through the reduction of vector populations have been successful in some areas of Latin America, effective control measures are lacking in most regions of endemicity (Nowicki *et al.*, 2006).

During the acute phase, the parasite infects a wide variety of tissues including the viscera and central nervous system. In the chronic phase, lesions in the central, peripheral autonomic and enteric nervous systems lead to widely diverse disorders of the hollow muscular organs, including the heart, esophagus and colon (Koberle, 1968), which is thought to be due, at least in part, to inflammatory related mechanisms (Corbett *et al.*, 2001; da Silveira *et al.*, 2005b; Lemos *et al.*, 1998).

Among inflammatory cells, macrophages, eosinophils and mast cells may be of particular importance in the pathogenesis of megacolon, as they can produce a great number of substances involved in host defence and gut physiology, including cytokines and neurotrophins (Kariyawasam & Robinson, 2006; Noga *et al.*, 2005). In this study we described the distribution of macrophages, eosinophils and mast cells in the colon of chagasic patients, and we further developed quantitative analyses in order to investigate the correlation between the concentration of these inflammatory cells and the area of fibrosis.

MATERIAL AND METHODS

Patients and Samples

Samples of full thickness colon wall tissue were obtained from 8 chagasic patients with megacolon, 8 chagasic patients without megacolon and 10 non-infected individuals submitted to necropsy or surgical procedures at Faculdade de Medicina do Triângulo Mineiro (Uberaba, Minas Gerais, Brazil). Patients did not receive any parasite-specific treatment. Informed consent was obtained from the patient or family members prior to tissue procurement. This work was approved by UFMG Research Ethics Committee.

Serological tests indicative of Chagas' disease (complement fixation, hemagglutination, and immunofluorescence tests) were positive in all patients studied. All of the patients had left the endemic area more than 20 years before the tissue collection, and during that time, patients without megacolon did not present any symptom related to digestive disease. All patients originated from Uberaba, MG, Brazil, where the natural transmission of Chagas' disease was interrupted more than 20 years ago, and they had never received blood transfusions. The patients with and without megacolon had mean ages of 57 ± 10 years, and 55 ± 14 years respectively. The presence of megacolon was established based in clinical data reporting colon obstruction, and from radiological studies. Manometric studies of megacolon demonstrated decreased peristalsis and incomplete relaxation of the anal sphincter. These abnormalities precede clinical symptoms and dilatation seen by radiographic studies.

The control group was composed of non-infected individuals, as indicated by negative serology specific for Chagas' disease. Non-infected individuals were also from the state of Minas Gerais and had a mean age of 54 ± 20 years.

Histology and peroxidase immunohistochemistry

Tissue samples were collected from the recto-sigmoid region. Each specimen was fixed in 4% neutral buffered formaldehyde solution and embedded in paraffin for immunohistochemistry studies. Sections of 7µm were deparaffinized using xylene, and rehydrated through graded alcohols. Some sections were then stained for standard histology, using haematoxylin and eosin (H&E) (eosinophils), toluidine blue (mast cells) and Gomori trichrome (fibrosis), while others were prepared for immunohistochemistry. For immunohistochemistry, endogenous peroxidase was inhibited by incubation with 1% hydrogen peroxide and 30% absolute methanol for 30 min. The slides were then incubated with 2% normal swine serum (NSS) (Sigma, USA) in phosphate buffered saline (PBS) for 15 min and subsequently with the anti-CD68 monoclonal antibody (DAKO, KP1, 1:100, USA). Following this, the tissue sections were incubated with peroxidase-conjugated rabbit anti-mouse antibodies (DAKO) for 45 min, and peroxidase activity was detected by incubation with 3,3'-diaminobenzidine (Sigma) and hydrogen peroxide for 10 min. Slides were counterstained with Gill's haematoxylin (Sigma), dehydrated in graded alcohols, and mounted in synthetic mounting media. Negative control slides without primary antibody were performed for each case.

Cell quantification and morphometric studies

Enumeration of eosinophils and mast cells was performed in the enteric plexuses (submucosal and myenteric plexuses) and muscle layers, by counting 20 randomly-selected fields (total area of 1066µm^2) on a single slide per patient.

Morphometric studies of macrophages were performed by image analysis (Kontron KS300 v. 2.0), by measuring CD68-IR areas on 20 randomly-selected fields (total area of 1066μm^2) in each colon segment (submucosal plexus, inner muscular, myenteric plexus and outer muscular) on a single slide per patient.

Analysis of fibrosis was performed in 20 alternate fields in each muscle layer (total area of 1066μm^2 per muscle layer) on a single slide per patient. For the quantitative analysis, images were obtained using a graphical measuring grid (Kontron KS 300) connected to an automatic image analysis system. All analysis was performed using the 40x objective lens.

Statistical analyses

Statistical analysis among the different groups was performed. Data were expressed as the mean ± SD. Differences between groups were evaluated by one-way ANOVA, and the relation between the inflammatory cells and fibrosis occurrence were evaluated by Simple Linear Regression tests. Significance was defined at $P \leq 0.05$.

RESULTS

Analyses of eosinophils and mast cells

Eosinophils were observed in the colon sections of individuals from all groups analysed, dispersed through the neuronal plexuses regions. In chagasic patients, they were also observed in the muscle layers, associated with inflammatory foci and fibrosis (Figure 1A). When pairs of groups were analysed together, it was observed that the numbers of eosinophils in all analyzed

layers were significantly greater in chagasic patients with megacolon than in the other two groups. There was no significant difference between non-infected individuals and chagasic patients without megacolon (Table 1).

Mast cells were observed diffusely dispersed through areas of fibrosis, independently of the group analysed. In chagasic patients with megacolon, they were also observed forming isolated clusters or even surrounding inflammatory foci (Figure 1B). Mast cell counting and statistical analyses revealed significant increased number of these cells in the colon of chagasic, when it was compared to non-infected group or even to patients without megacolon (Table 2).

Morphometric analyses of CD68-IR macrophage-occupied areas

Morphometric analysis of anti-CD68 pre-treated colon slices revealed a significant increase in the macrophage-occupied area in chagasic patients, compared with non-infected group, as well as with patients without megacolon (Figure 1C). In the myenteric plexus, the macrophage-occupied area in patients without megacolon was increased when compared to non-infected individuals, what suggests a correlation between the concentration of macrophage and the progression of the disease (Table 3).

Morphometric analyses of fibrosis

Analyses of Gomori trichromic-stained sections revealed that chagasic patients with megacolon present increase endomysial and perimysial connective tissue deposition and frequent fibrosis foci. Collagen deposition was rarely observed in the muscle layers of the other two groups analysed, being represented most by endomysial or perimysial connective tissue.

Morphometric analyses of stained sections revealed significant enlargement of the area occupied by fibrosis in patients with megacolon, when they were compared with non-infected controls or even with patients without megacolon (Table 4).

Analyses of correlation between inflammatory cells and fibrosis

The statistical test Simple Linear Regression was used to evaluate the correlation between the number of inflammatory cells and the area of fibrosis. Data analyses demonstrated that the presence of all studied inflammatory cells have a directly correlation with the fibrosis occurrence (Figure 2), what is easily observed in chagasic patients with megacolon group ($P<0.05$).

DISCUSSION

The data presented in this study revealed that the development of megacolon in chronic Chagas disease is associated with increasing concentrations of mast cells, eosinophils and macrophages in the organ. We could speculate that the increased concentrations of these inflammatory cells in the organ of chagasic patients could be consequence of the megacolon development. This pathological process is characterized by faecal accumulation in the intestine lumen, which may compress the mucosa and cause ischemia, with subsequent regressive cellular injuries and inflammation. The inflammatory process can reach the submucosa and, ultimately the muscle layers and myenteric plexus (Tafuri *et al.*, 1971). The accumulation of some inflammatory cells in patients with megacolon could be consequence of this process, in which the chronic inflammation induces cellular injury and necrosis. Studies carried out by Kobayashi (Kobayashi *et al.*, 2002) and Noga (Noga *et al.*, 2003) demonstrated that these cells are capable

of producing, storing and secreting neurotrophins, essential for survival of neurons (Hashimoto *et al.*, 2005; Mendell *et al.*, 1999; Micera *et al.*, 2003), and cytokines that participate in tissue regeneration (Hirschberg *et al.*, 1994; Kiefer *et al.*, 2001; Lakatos & Franklin, 2002; Slawinska *et al.*, 2000; Smythies *et al.*, 2005). In this context, we would speculate that the increased density of eosinophils, mast cells and macrophages in chagasic megacolon, could be view as an attempt of the intestine homeostasis reestablishment. The significant presence of those cells in the colon of non-infected individuals, imply their role in the maintenance of intestinal physiology, as it has been well described in the literature (Levy *et al.*, 2001; Straumann & Simon, 2004).

It is well known by the literature that macrophages, eosinophils, as well as mast cells, in the context of any inflammatory process, secrete cytokines such as IL-1, TNF-α and IL-6, which have the capacity to activate immune-cytotoxic mechanisms (Daryani *et al.*, 2003). Moreover, eosinophils and macrophages, by themselves, have cytotoxic potential by producing, when activated, substances such as nitric oxide and free radicals (Geboes, 1994). In view of the observations above, the data presented in this study point to the participation of these inflammatory cells in the tissue lesions that are associated with the development of chronic chagasic megacolon. Increased concentrations of eosinophils and macrophages have been already described in oesophagus (da Silveira *et al.*, 2005a) and hearts (Milei *et al.*, 1991) of chagasic patients, suggesting a common mechanism in the development of the different syndromes associated with chronic Chagas disease.

Considerable research has been developed with the assumption that interactions between immune cells and fibroblasts are paramount in the genesis of fibrosis (Otte *et al.*, 2003; Powell, 1994). Among these cells, eosinophils, mast cells and macrophages have been received special attention. They are associated with fibrosis in various chronic pathologies, such as Chron disease (Xu *et al.*, 2004), and *in vitro* studies have demonstrated that they interact with fibroblast in a

manner that leads to fibroblast activation and subsequent extracellular fibrosis (Musso *et al.*, 1999; Otte *et al.*, 2003).

In chagasic megacolon, enlarged areas of fibrosis and increased density of mast cells have been already described (Pinheiro *et al.*, 2003; Tafuri *et al.*, 1971), but to our best knowledge, this is the first report demonstrating a statistically significant correlation between eosinophils, mast cells and macrophage concentration and collagen deposition in both muscle layers. Future studies on the definitions of mediators involved in the recruitment of such inflammatory cells to the site of inflammation can help define therapeutic targets in order to avoid or delay the development of megacolon in Chagas disease.

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Quantification of eosinophils associated with the enteric plexuses and muscle layers from chagasic patients with and without megacolon (number of cells / mm^2)

	Non-infected individuals	Chagasic patients without megacolon	Chagasic patients with megacolon
Submucousal plexus	18 ± 3	27 ± 8	[ab]66 ± 23
Myenteric plexus	9 ± 2	17 ± 6	[ab]43 ± 15
Inner muscle layer	8 ± 2	15 ± 4	[ab]27 ± 3
Outer muscle layer	7 ± 1	10 ± 2	[ab]26 ± 6

Table 1 – Mean number and standard deviation of eosinophils in the enteric plexuses and muscle layers from chagasic patients' colon. (a) Statistically significant differences between this group and non-infected individuals; (b) Statistically significant differences between this group and chagasic patients without megacolon ($P<0.05$). Total analysed area of 1066µm^2 from each field for all patients was analyzed.

Quantification of mast cells in the enteric plexuses and muscle layers from chagasic patients with and without megacolon (number of cells / mm^2)

	Non-infected individuals	Chagasic patients without megacolon	Chagasic patients with megacolon
Submucousal plexus	56 ± 13	83 ± 17	[ab]195 ± 24
Myenteric plexus	18 ± 2	22 ± 5	[ab]47 ± 13
Inner muscle layer	16 ± 2	18 ± 4	[ab]35 ± 6
Outer muscle layer	23 ± 2	17 ± 5	[ab]30 ± 4

Table 2 – Mean number and standard deviation of mast cells in the enteric plexuses and muscle layers from chagasic patients' colon. (a) Statistically difference between this group and non-infected individuals; (b) Statistically differences between this group and chagasic patients without megacolon ($P<0.05$). Total analysed area of 1066µm^2 from each field for all patients was analyzed.

Quantification of macrophage CD68-IR area in colon of chagasic patients with and without megacolon and non-infected individuals

	Non- infected individuals	Chagasic patients without megacolon	Chagasic patients with megacolon
Submucous plexus	116 ± 33	180 ± 52	[a]228 ± 64
Myenteric plexus	53 ± 21	[a]127 ± 34	[a]174 ± 70
Inner muscle layer	25 ± 9	24± 9	[ab]118 ± 55
Outer muscle layer	20 ± 9	26 ± 7	[ab]164 ± 63

Table 3 – Mean number and standard deviation of macrophage CD68-IR area in the colon of chagasic patients. (a) Statistical differences between this group and non-infected individuals. (b) Statistical differences between this group and chagasic patients without megacolon group ($P<0.05$). Total analysed area of 1066μm^2 from each field for all patients was analyzed. Reactive tissue area (μm^2) per total tissue area (mm^2).

Quantification of proportional area occupied by fibrosis in colon of chagasic patients with and without megacolon and non-infected controls			
	Non- infected controls	Chagasic patients without megacolon	Chagasic patients with megacolon
Inner muscle layer	42 ± 14	70 ± 19	[ab]202 ± 37
Outer muscle layer	35 ± 11	63 ± 24	[ab]151 ± 42

Table 4 – Mean number and standard deviation of fibrotic area of muscle layers from chagasic patients' colon. (a) Statistically differences between this group and non-infected individuals. (b) Statistically differences between this group and chagasic patients without megacolon ($P<0.05$). Total analysed area of 1066μm^2 from each field for all patients was analyzed. Affected tissue area (μm^2) per total tissue area.

Figure 1 – Characterization of eosinophils, mast cells and macrophages in the colon of chagasic patients with and without megacolon and non-infected individuals. (A) Hematoxylin-eosin section: eosinophils can be easily observed (arrow) in the colon associated with fibrosis and inflammatory mononuclear cells. (B) Toluidine blue section: chagasic patients with megacolon shown clusters of mast cells associated with fibrosis foci (arrow). Others inflammatory cells were not seen near of mast cells clusters. (C) Immunohistochemistry to macrophages CD68-IR: Macrophages can be easily seen in the muscle layers and near of inflammatory foci in the colon of chagasic patients with megacolon.

Figure 2: Relation among the number of eosinophils (A), mast cells (B) macrophages area (C) and fibrosis occurrence in the colon of chagasic patients with megacolon. Data analyses demonstrated that the presence of all studied inflammatory cells have a directly relation with the fibrosis occurrence in these chagasic patients ($P<0.05$).

ANEXO 3

Date: Nov 07, 2006

To: "Alexandre da Silveira" alec@icb.ufmg.br

From: "Digestive Diseases and Sciences" dustevichmw@msx.upmc.edu

Subject: #DDAS2611R - Decision on your manuscript

Dear Alexandre da Silveira:

On behalf of the Editorial Board, I am pleased to inform you that your manuscript, "NEUROCHEMICAL CODING OF THE ENTERIC NERVOUS SYSTEM IN CHAGASIC PATIENTS WITH MEGACOLON" has been accepted for publication in Digestive Diseases and Sciences.

Thank you for submitting this manuscript to us. It is papers as yours that will maintain the excellence of our Journal.

Sincerely,

Richard L Wechsler, MD
Editor-in-Chief
Digestive Diseases and Sciences

NEUROCHEMICAL CODING OF THE ENTERIC NERVOUS SYSTEM IN CHAGASIC PATIENTS WITH MEGACOLON

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Acknowledgements: This work was supported by funds from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Ministério da Educação, Brazil and the National Health and Medical Research Council of Australia (grant 400020)

Running Read: Neurochemical coding of the enteric nervous system in chagasic patients with megacolon

ABSTRACT

Purpose: Neuronal destruction had been considered the hallmark of pathogenic mechanisms in chagasic megacolon. The characterization of neuropeptides in the enteric nervous system from chagasic patients with megacolon could elucidate some aspects about the development of this syndrome. In the present work we demonstrate the changes of expression in neuropeptides and neurochemical markers presents in the neuronal plexuses from the colon of chagasic patients with megacolon.

Methods: Sections of frozen tissue samples were immunohistochemically labelled for anti-calretinin, cChaT, substance P, VIP, NOS and NPY. Immunoreactivity was observed by the use of confocal microscope.

Results: Our results demonstrated that in chagasic patients with megacolon, the inhibitory motor neurons (VIP and NOS immunoreactive) are preferentially destroyed by the *T. cruzi* and/or inflammatory process.

Conclusions: These results suggest a selective destruction of enteric neurons in the colon of chagasic patients with megacolon, pointing to an important discovery in the mechanism of the Chagas disease pathogenesis.

Keywords: Chagas'disease, chagasic megacolon, enteric nervous system, neurochemical markers, neuropeptides

INTRODUCTION

Chagas' disease is a consequence of infection with the parasite, *Trypanosoma cruzi (T. cruzi)*. Its acute phase is relatively mild, but 10-50% of those affected develop chronic disease. The infection is endemic to parts of south and central America, and more than 100 million people of these regions are at risk of infection. Numerous migrants from these regions also suffer from the disease (1).

The acute phase of the disease can last from weeks to months and typically is asymptomatic or associated with fever and other mild non-specific manifestations. The death rate for persons in this phase is about 10%. Chronic Chagas' disease is characterized by cardiopathy, megaesophagus and / or megacolon (2,3).

In the chronic phase, lesions in the central and peripheral autonomic nervous systems, including the enteric nervous system, lead to widely diverse disorders of the hollow muscular organs, including the heart, esophagus and colon (4). Although there is little controversy that *T. cruzi* is the causative agent, there is also little known about why patients have selective organ involvement. Understanding the mechanisms for this selective clinical presentation of Chagas' disease may lead to a better understanding, not only of this disorder but of other functional bowel diseases. In the chronic phase of the disease the most typical pathologic feature is a reduction in the number of nerve cells in both the submucosal and myenteric plexuses (5).

Little is known about the types of neurons that are affected by this neuropathic process. Moreover, no study has previously compared the relative effects of Chagas' disease in dilated and non-dilated portions of the colon. Demonstrating immunoreactivity in cell bodies, we investigated the neurochemical coding in the human ENS from Chagasic patients utilizing some markers of specific neurons, calretinin, neuropeptide Y (NPY), choline acetyl transferase

(cChAT), substance P (SP), vasoactive intestinal peptide (VIP) and nitric oxide synthase (NOS). These would then allow more detailed assessment of changes in the neurochemistry of enteric neurons in the chagasic megacolon development.

METHODS

Patients and tissue collection

Colon tissue samples were collected from twelve patients. These patients were classified in two groups: non-infected individuals (n=4) and chagasic patients with megacolon (n=8). Certain samples from patients with megacolon had non-dilated portion and dilated portion. Both portions were randomly found in the colon and they were very similar between patients. For that reason, we analyzed both portions from these samples, classifying chagasic patients with megacolon in two groups: non-dilated portion (NDP) and dilated portion (DP) from chagasic patients with megacolon. Both portions were randomly current in the colon. Patient's details are shown in Table 1.

Reasons for tissue resection were colon complications caused by Chagas' disease or colon carcinoma in non-infected individuals. All tissues were collected with patient' consent and the collection and use were approved by the Human Ethics Committee of the Universidade Federal de Minas Gerais.

Colon samples were collected in phosphate-buffered saline (PBS). These were fixed overnight at 4°C in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer (pH 7.0). The next day, tissue was cleared of fixative with dimethylsulfoxide (DMSO) with up to 6x10 min washes followed by 3x10 min washes in PBS. The tissues were then placed in PBS-sucrose-azide (PBS containing 0.1% sodium azide and 30% sucrose as a cryoprotectant) and

stored at 4°C. The following day, small segments of tissue were transferred to a mixture of PBS-sucrose-azide and OCT compound (Tissue Tek, Elkhart, IN, USA) in a ratio of 1:1 for a further 24 h before being embedded in 100% OCT. Sections of 12 μm thickness were cut and collected on microscope slides and left to dry for 1 h at room temperature.

Immunohistochemical investigation

Double-staining immunohistochemistry was conducted combining the HuC/HuD antibody with other antibodies listed in Table 2. Sections were first incubated in 10% normal horse serum (NHS) plus 1% triton X-100 for 30 min. Incubation with primary antibodies was carried out for 24 h at 4°C with diluted antiserum containing 10% NHS. Double-labelling was achieved using a combination of HuC/HuD and neuronal active peptides or neuronal markers antibodies. Following incubation in primary antiserum, preparations were rinsed in PBS (3x10 min) and then incubated for 1 h at room temperature with secondary antibodies (Table 3). Further 3x10 min washes in PBS were made before tissue was mounted in DAKO fluorescence mounting medium (DAKO, California, USA).

Quantification procedures

Anti-HuC/HuD was used as a general neuronal marker to determine the total number of cell bodies in the nervous plexuses of the human colon. In each tissue sample, double-stained sections for HuC/HuD and a specific neuronal marker were performed. Sections through ganglia that contained at least three nerve cells in the plane of section were selected randomly, in a meander-like fashion, until a total of 80 neurons was analysed in each ganglionated plexus

(submucosal and myenteric plexuses). The only criterion for selecting a profile of a ganglion was that it contained at least three stained neurons. Using confocal laser scanning microscopy (Bio-Rad MRC 1000 attached to a Nikon diaphot 300, equipped with a krypton-argon laser, American Laser Corporation, Salt Lake City, UT), single optical section images on the same focus plane were created in the ganglia by applying two different excitation wave lengths. The filter settings were 594 nm excitation and 647 nm. A 40 times oil immersion objective lens (numerical aperture 1.3) was used, the zoom factor was set to 1.0 in all scanning sessions. Pictures were prepared using Confocal Assistant 4.02, and CorelDraw 13. For counting of reactive neurons, HuC/HuD and specific neuronal marker pictures were opened sequentially. Stained neurons were outlined with two differently marker pens. Thereafter, all neurons on the sheet marked with one or two colours were counted.

Statistics

Statistical analysis among the different groups was analysed by one-way ANOVA. Differences were considered statistically significant at $P<0.05$.

RESULTS

Populations of immunoreactive nerve cells in nervous plexuses of the colon

With the HuC/HuD antibody, the architectures of the neuronal plexuses were clearly visible in the sections. HuC/HuD immunoreactive (IR) cell bodies per ganglion profile were almost identical between non-infected individuals and the NDP from chagasic patients with

megacolon, while in DP from chagasic patients with megacolon there were fewer neuronal bodies per section through each ganglion. Details of the cell counts obtained with each of the antisera are shown in Table 4.

Double labelled data were based on calculations of immunoreactive cell bodies. The proportions of the neurochemically identified neuronal subpopulations were expressed relative to the number of HuC/HuD-IR cell bodies in the submucosal and myenteric plexuses.

The analyses of neurons from submucosal plexus of the colon showed that the majority of neurons in non-infected individuals and NDP from chagasic patients with megacolon were VIP-IR (47% and 43%), while DP from chagasic patients with megacolon showed cChAT-IR as the majority (26%). The numbers of calretinin, cChAT, and NPY-IR neurons was not changed in chagasic patients in relation with the non-infected individuals. A relatively small population of neurons in the submucosal plexus was SP-IR in non-infected group and NDP from chagasic patients with megacolon (8% and 9%). However, DP from chagasic with megacolon presented an increased number of SP-IR neurons (22%). By the other side, NOS and VIP-IR neurons were very rarely encountered in DP from chagasic with megacolon, showing a considerable decrease of these neurons in comparation with the other groups (Table 5).

The analyses of neurons from myenteric plexus of the colon showed that the majority of neurons in all groups were cChAT-IR. The numbers of calretinin (Figure 1, A and A'), NPY (Figure 1, B and B') and cChAT-IR neurons (Figure 1, C and C') was not changed in chagasic patients in relation with the non-infected individuals. As in the submucosal plexus, non-infected group and NDP from chagasic patients with megacolon showed a relatively few SP-IR neurons in the myenteric plexus (5% and 7%) (Figure 1, D). However, DP from chagasic with megacolon presented an increased number of SP-IR neurons (20%) (Figure 1, D'). On the other hand, NOS (Figure 1, E) and VIP-IR (Figure 1, F) neurons were very rarely encountered in DP from chagasic

with megacolon (Figure 1, F and F'), showing a considerable decrease of these neurons in comparation with the other groups (Table 6). For all antigens used in this study there was no correlation between the age of patients and the number of immunoreactive cell bodies.

DISCUSSION

American trypanosomiasis still affects millions of people in Latin America and causes 50.000 deaths annually (6). The megacolon is one of the clinical manifestations of this chronic disease. The rectum-sigmoid transition of the colon dilates more than any part of the large bowel and is usually affected by complications such as faecal impaction and sigmoid volvulus (4). The exact mechanism of the neural damage is not clearly understood. Furthermore, the pathophysiologic mechanism by which the neuronal destruction causes visceral dilation has been the subject of much discussion. Neuronal lesions may impair motility involved with colonic transit, colonic outlet mechanisms, or tone (5).

Chagas' disease is often described as a disorder of outlet obstruction similar to Hirschsprung's disease. Although both diseases cause distal colonic dilation and myenteric neuropathy, the lesion in Hirschsprung's disease is localized to the distal rectum, whereas the neuropathy in Chagas' disease is more diffuse (7). The more widespread distribution suggests that the neuropathic process may also affect afferent pathways. Denervated smooth muscle is abnormally responsive to stimuli (8) leading to increased contractility; a small stimulus will then provoke strong irregular and often complex contractions. In the colon, the first effect of denervation is loss of muscular coordination, with the degree of functional disturbance varying according to its extent. The loss of coordinated peristalsis and sphincter function delays passage of intestinal contents (8,9).

The complexity of the enteric nervous system is reflected by a diversity of phenotypes of the enteric neurons in both plexuses. Colocalisation studies using different neuronal markers are crucial to determine subpopulations of neurons (10-12). Distinct neuronal markers of subclasses of neurons in chagasic patients with megacolon have not been reported previously. In this study, a double-labelling immuno-fluorescent technique using antibodies was employed to determine the population of neuronal bodies in the enteric plexuses of the colon from chagasic patients with megacolon. Calretinin have been used to identify IPANs (Intrinsic primary afferent neurons), cChAT and SP to identify as excitatory motor neurons, NPY to identify inter-neurons and, NOS and VIP to identify inhibitory motor neurons in the human colon (10, 12-14).

The total proportion of calretinin, cChAT and NPY-IR neurons was not modified in chagasic patients compared with non-infected individuals. These data suggest that there is no alteration of IPANs, of cChAT-IR excitatory motor neurons and inter-neurons NPY-IR in chagasic patients with megacolon. However, the increase in SP-IR neurons was observed in both enteric plexuses of the colon in chagasic patients with megacolon. Elevated levels of SP have been reported in the rectum and colon of UC patients, and correlate with disease activity (15,16). Autoradiographic studies demonstrated a 1.000-fold upregulation of SP binding sites in the lymphoid follicles and submucosal vasculature of patients with IBD (17).

What might be the clinical relevance of the SP increase in chagasic megacolon? Firstly, it was reported that SP may play an important role in the pathophysiology of the ulcerative colitis (UC) and Crohn’s disease (CD) (18). In an animal model, inflammation induced an increase in SP synthesis in myenteric neurons (19). It is possible that these increased SP level could result in a net increase in SP that might contribute to uncontrolled inflammation, as is observed in chagasic patients with megacolon (5).

Our data showed clearly that NOS and VIP-IR neurons in both neuronal plexuses of the colon is decreased in DP from chagasic patients with megacolon when compared with non-infected individuals. Thus, there was a surprise about the selectivity of the neuropathy. This represents, to our knowledge, the first report on NOS and VIP alterations in chagasic megacolon. The depletion of NOS and VIP containing neurons may prevent smooth muscle relaxation in the damaged colon of these patients. This injury may affect transit, colonic tone, or muscular relaxation responsible for decreased outlet resistance. Similarly, NOS and VIP alterations play also a role in Hirschsprung's disease, a congenital absence of Meissner's and Auerbach's plexuses of the colon (20,21). This disease results in the inability of the involved colon and internal anal sphincter to relax, inducing a mechanical obstruction and an increase in the amount of acetylcholinesterase containing nerve disease occurs. Therefore, the presence or absence of nitric oxide synthase-containing neurons is crucial in the failure of internal anal sphincter relaxation that characterizes Hirschsprung's disease (22-24).

In the colon, NO and VIP nerves are responsible for the associated relaxation seen during peristalsis and internal anal sphincter relaxation. Involvement of NO in nerve-mediated relaxation of the human internal anal sphincter is suggested by the behaviour of sphincter tissue *in vitro* (25). Isolated strips of human internal anal sphincter smooth muscle relax in response to inhibitory nerve stimulation, and this effect is mimicked by application of NO from an exogenous source. In addition, neurogenic relaxation can be abolished by inhibitors of nitric oxide synthase, the enzyme that catalyses the formation of NO, as well as by agents that scavenge NOS from extracellular media (25,26). Recent data from mammalian models have suggested that the small intestine and colon may be subject to tonic neural inhibition mediated by NO (27,28). In normal colon, local distension within the lumen of the colon or distal rectum induces a neural reflex that produces smooth muscle contraction proximal and relaxation distal to this site of distension (29).

Morphologic evidence indicates that in the human colon myenteric plexus NOS is present within a discrete but substantial subpopulation of enteric neurons (30). The neurons have the correct projections and neurochemistry to be the inhibitory motor neurons that mediate the descending inhibitory reflex in intestinal peristalsis. The morphologic data suggest that, NO-containing neurons and their processes have the appropriate morphologic characteristics and distribution to serve as inhibitory motor nerves that convey information between the rectum and the internal anal sphincter (31). It is noteworthy that NO is the principal mediator of the inhibitory reflex, and Chagas' disease may be accompanied by achalasia of the internal anal sphincter. Attempts to correct this imbalance may contribute to ameliorating the symptoms of these patients.

Internal anal sphincter relaxation has already been shown to be possible with the use of topical glyceryl trinitrate ointment, a nitric oxide donor, and this treatment has been applied to anal fissures with relatively good results, producing decreased anal pressure and healing the fissures (32-34). In the future, manipulation of NO through genetic engineering, viral transfection, or NO-releasing medication may prove valid to treat chagasic megacolon and others digestive diseases when the relaxation of the colon is compromised.

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Patients	*Gender*	*Age*	*Dilated portion length*	*Non-dilated portion length*	*Total length*
C8[a]	F	47	-	-	10 cm
C11[a]	M	59	-	-	12 cm
C22[a]	M	69	-	-	7 cm
C29[a]	F	54	-	-	11 cm
C1[b]	M	55	9 cm	-	9 cm
C2[c]	M	62	7 cm	4 cm	11 cm
C3[b]	M	55	11 cm	-	11 cm
C4[c]	M	65	3 cm	3 cm	6 cm
C5[b]	F	60	14 cm	-	14 cm
C6[b]	M	53	12 cm	-	12 cm
C7[b]	F	54	11 cm	-	11 cm
C12[c]	F	58	5 cm	2 cm	7 cm

Table 1: Patients and tissue morphological characteristics. (a) non-infected individuals; (b) chagasic patients with megacolon that presented only dilated portion; (c) chagasic patients with megacolon that presented dilated and non-dilated portions.

Primary antibodies list			
Antibody	**Source**	**Company**	**Dilution**
Anti-HuC/HuD	Mouse	Dako, USA	1:2000
Anti-Calretinin	Goat	Zymed Lab., USA	1:1000
Anti-cChaT	Rabbit	Dako, USA	1:200
Anti-SP	Rabbit	Peninsula Lab., UK	1:1000
Anti-NPY	Rabbit	Dako, USA	1:2000
Anti-NOS	Rabbit	Zymed Lab., USA	1:400
Anti-VIP	Rabbit	Peninsula Lab., UK	1:400

Table 2: Primary antibodies list

Secondary antibodies list		
Antibody	**Company**	**Dilution**
Alexa Donkey α Mouse 594	Mobitec, Germany	1:400
Alexa Donkey α Sheep 647	Mobitec, Germany	1:1000
Alexa Donkey α Rabbit 647	Mobitec, Germany	1:800

Table 3: Secondary antibodies list

Mean of immunoreactive neuronal cells bodies per ganglion in the colon from chagasic patients and non-infected individuals

	Non-infected individuals	NDP from chagasic patients with megacolon	DP from chagasic patients with megacolon
Submucosal plexus	11 ± 2	8 ± 2	4^{ab} ± 1
Myenteric plexus	18 ± 4	14 ± 3	6^{ab} ± 2

Table 4: Mean of immunoreactive neuronal cells bodies per ganglion in the colon from chagasic patients and non-infected individuals. The values are expressed as mean number of cell number ± S.D. (a) Statistically significant differences observed between this group and the non-infected individuals group. (b) Statistically significant differences observed between this group and the NDP from chagasic patients with megacolon. At least 10 ganglion were analysed for all patients ($P<0.05$).

Relative proportion of immunoreactive cell bodies in submucosal plexuses from chagasic patients with megacolon and non-infected individuals			
	Non-infected individuals	NDP from chagasic patients with megacolon	DP from chagasic patients with megacolon
HuC/HuD	100	100	100
Calretinin	28 ± 6	26 ± 4	21 ± 4
cChaT	32 ± 6	27 ± 6	26 ± 6
Substance P	8 ± 2	9 ± 2	22^{ab} ± 6
NPY	30 ± 7	29 ± 7	19 ± 5
NOS	10 ± 3	9 ± 3	1^{ab} ± 1
VIP	47 ± 8	43 ± 8	12^{ab} ± 4

Table 5: Relative proportion of immunoreactive cell bodies in submucosal plexus from chagasic patients with megacolon and non-infected individuals. (a) Statistically significant differences observed between this group and non-infected individuals; (b) Statistically significant differences observed between this group and non-dilated portion (NDP) from chagasic patients with megacolon. The neuronal count was realized by the analyses of at least 80 neuronal bodies per patient in the submucosal plexus ($P<0.05$).

Relative proportion of immunoreactive cell bodies in myenteric plexuses from chagasic patients with megacolon and non-infected individuals			
	Non-infected individuals	NDP from chagasic patients with megacolon	DP from chagasic patients with megacolon
HuC/HuD	100	100	100
Calretinin	40 ± 7	34 ± 7	30 ± 4
cChaT	57 ± 8	45 ± 7	42 ± 8
Substance P	5 ± 2	7 ± 2	20^{ab} ± 5
NPY	10 ± 3	9 ± 2	7 ± 2
NOS	20 ± 6	16 ± 4	4^{ab} ± 2
VIP	25 ± 6	22 ± 6	5^{ab} ± 1

Table 6: Relative proportion of immunoreactive cell bodies in myenteric plexus from chagasic patients with and without megacolon and non-infected individuals. (a) Statistically significant differences observed between this group and non-infected individuals; (b) Statistically significant differences observed between this group and non-dilated portion (NDP) from chagasic patients with megacolon. The neuronal count was realized by the analyses of at least 80 neuronal bodies per patient in the submucosal plexus ($P<0.05$).

Figure 1: Immunohistochemical demonstration of neurochemical coding in human myenteric ganglia from non-infected individual and chagasic patients with megacolon. Double immunohistochemistry for HuC/HuD (red) and neurochemical marker (green). There are no difference between the number of calretinin-IR cell bodies in non-infected individual (A) and chagasic patient with megacolon (A'). The same is observed in the number of NPY (B and B') and cChAT-IR cell bodies (C and C'). Immunohistochemistry for SP revels that chagasic patients with megacolon presented more numerous SP-IR cell bodies (D') in relation with the non-infected individuals (D). By the other side, only very few cell bodies in chagasic patients with megacolon were VIP and NOS-IR (E' and F') in relation with non-infected individuals (E and F).