DÉBORA D'ÁVILA REIS

IMUNOPATOLOGIA DA DOENÇA DE CHAGAS CRÔNICA: ANÁLISE DA EXPRESSÃO IDIOTÍPICA EM ANTICORPOS ANTI-EPIMASTIGOTA E ESTUDO IMUNOHISTOQUÍMICO DA LESÃO CARDÍACA

Tese apresentada ao curso de Pós-graduação do Departamento de Bioquímica e Imunologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito básico para obtenção do titutlo de Doutor.

Orientador: Prof. Giovanni Gazzinelli

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Dedico esta tese ao Bernardo

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O Colegiado do Curso e a Comissão de Avaliação aprovaram o seguinte formato de tese:

-Coletânea de três (3) trabalhos publicados que atendam às exigências do Regulamento do Curso

-Em pelo menos um destes trabalhos o aluno tem que ser o primeiro autor

-Não poderão ser estes trabalhos utilizados para outra tese de doutorado

-O conjunto de tese deverá conter uma Introdução **ou** apresentação dos trabalhos, seguido de uma conclusão global.

O objetivo básico deste trabalho foi estudar alguns aspectos relacionados com a imunopatologia da doença de Chagas, com ênfase na forma clínica crônica cardíaca. Abordamos este assunto sob dois ângulos:

- a nível do sangue periférico, comparando a expressão idiotípica em anticorpos de pacientes chagásicos crônicos Cardíacos e Indeterminados

- a nível da lesão cardíaca, fazendo um estudo imunohistológico dos vários componentes do sistema imune atuantes no local.

Esta dissertação de tese se originou dos seguintes trabalhos:

Trabalho #1

REIS, D.d'A.; GAZZINELLI, R.; GAZZINELLI, G.; COLLEY, D.G. Antibodies to *Trypanosoma cruzi* Express Idiotypic Patterns that can differentiate between Patients with Asymptomatic or Severe Chagas' Disease. *J. Immunol. 150 (4)*: , 1993.

Trabalho #2

REIS, D.d'A.; JONES, E.M.; TOSTES, S.; LOPES, E.R.; GAZZINELLI, G. COLLEY, D.G.; McCURLEY, T. Characterization of Inflammatory Infiltrates in Chronic Chagasic Myocardial Lesions: Presence of TNF-alpha+ Cells and Dominance of Granzyme A+, CD8+ lymphocytes. *Am. J. Trop. Med. Hyg. (in press)*

Trabalho #3

REIS, D.d'A.; JONES, E.M.; TOSTES, S.; LOPES, E.R.; CHAPADEIRO, E.; GAZZINELLI, G.; COLLEY, D.G.; McCURLEY, T. Expression of MHC antigens and Adhesion Molecules in Hearts of Patients with Chronic Chagas' Disease. *Am. J. Trop. Med. (in press)*

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APRESENTAÇÃO DOS TRABALHOS

Assim como diversas outras doenças parasitárias, a infecção humana pelo *Trypanosoma cruzi* se manifesta sob várias formas clínicas. As manifestações da fase aguda - dentre elas citam-se febre, linfadenite, meningoencefalite e miocardite - estão em geral relacionadas com a grande concentração do parasita no sangue ou nos diferentes tecidos. Todos estes sintomas geralmente desaparecem completamente em poucos meses (BRENER, 1987). A infecção crônica se caracteriza por baixa parasitemia (BRENER & KRETTLI, 1990) e baixa concentração dos parasitas nos tecidos (ANDRADE & RAMALHO, 1966; ANSELMI e cols., 1966; LOPES e cols., 1975; PALACIOS-PRU e cols., 1989; BARBOSA & ANDRADE, 1984). A sintomatologia da fase crônica pode ser decorrente de anormalidades no sistema digestivo e/ou de alterações cardíacas. Estas últimas incluem cardiomegalia, insuficiência cardíaca congestiva e alterações do sistema de transmissão do coração, sendo estas diagnosticáveis por raio X e eletrocardiograma (BRENER, 1987).

Um dos grandes enigmas no estudo da doença de Chagas humana é o longo período assintomático que permeia a evolução da fase aguda para a fase crônica sintomática. Os sintomas inerentes à miocardite da fase aguda em geral desaparecem em um curto espaço de tempo e só se manifestam após 10-30 anos (PRATA e cols., 1985) . Alguns indivíduos infectados, depois de sobreviverem à fase aguda, não chegam a apresentar nehuma sintomatologia durante toda a vida. Aos exames de raio X e eletrocardiograma estes pacientes não demonstram nenhuma alteração e são por isto denominados pacientes Indeterminados (DIAS, 1989).

A forma mais importante da doença de Chagas crônica é a forma clínica Cardíaca, pela sua prevalência, morbidade e mortalidade. Ela acomete cerca de 20% dos indivíduos infectados, incapacitando-os para o trabalho ou levando-os à morte (BRENER, 1987).

Acredita-se que vários fatores relativos ao hospedeiro e ao parasita atuem conjuntamente influenciando a imunopatogenia da cardiopatia chagásica crônica humana (KUHN, 1981). Dentre eles citam-se variabilidade genética do hospedeiro (GUSMÃO, 1985; LLOP e cols., 1988), re-infecções (MACEDO, 1973), tropismo do *T. cruzi* pelas miocélulas (KREUTER e cols., 1988) e diversidade de cepas do parasita (SCHLEMPER e cols., 1983; ANDRADE, 1990).

Dentre os componentes etiológicos da cardiopatia chagásica, um deles é comprovadamente de natureza imunológica. Análises histológicas de cortes de tecidos de coração provenientes de autópsia ou biópsia de pacientes portadores de cardiopatia chagásica crônica mostram infiltrados proeminentes de células inflamatórias e um intenso processo de fibrose. O processo inflamatório, em geral, acomete todo o miocárdio, incluindo os sistemas de condução. Na emergência dos grandes vasos pode ser notado um linfonodo aumentado de volume, o que reflete as reações inflamatórias em curso no local (ANDRADE & ANDRADE, 1979).

Vários estudos em animais experimentais e pacientes infectados com *T. cruzi* evidenciam um fenômeno de agressão de miocélulas por linfócitos locais. Análises histológicas em pacientes chagásicos (LOPES e cols., 1981), em cães (LOPES e cols., 1980) e coelhos (RAMIREZ, 1984) cronicamente infectados com *T. cruzi* bem como estudos à microscopia eletrônica em biópsias endomiocárdicas de cardiopatas chagásicos crônicos (COSSIO e cols., 1977), mostram células com morfologia de linfócitos intimamente aderidas a miocardiócitos em degeneração.

Estudos experimentais demonstram que a simples transferência de linfócitos T de camundongos crônicamente infectados para animais singeneicos não infectados, leva ao aparecimento de lesões no coração (LAGUENS e cols., 1981) e nos tecidos nervosos (SAID e cols., 1985) semelhantes àquelas observadas na fase crônica da infecção.

Devido à extensão da lesão e à escassez de parasitas no local, tem-se atribuido a patogenia da cardiopatia chagásica crônica a mecanismos autoimunes (TEIXEIRA, 1974; HUDSON, 1985; KIERSZENBAUM, 1985; PETRY & EISEN, 1989). Tem-se utilizado como argumentos a favor desta hipótese, a existência de autoanticorpos (COSSIO e cols., 1974; KHOURY e cols., 1979; SZARFMAN e cols, 1982; STERIN-BORDA e cols., 1988) e de linfócitos anti-miocárdio (MOSCA e cols., 1985; DE TITTO e cols., 1985, GATTAS e cols., 1988) no sangue periférico de pacientes chagásicos, bem como a demonstração de reatividade cruzada entre antígenos do *T. cruzi* e proteínas do hospedeiro (WOOD e cols. 1982; VAN VOOHIS & EISEN, 1989; MESRI e cols., 1990; LEVITUS e cols., 1991). Contudo na literatura não há nenhum trabalho que apresente provas de uma relação direta entre autoreatividade e patogenia na doença de Chagas crônica. É importante ressaltar que o sistema imune é naturalmente programado para reconhecer o "self" e que a mera semelhança entre epitopos do parasita e do hospedeiro não leva necessariamente a uma autoreatividade patológica.

Nos últimos anos tem-se dado ênfase à importância de interações idiotípicas na imunoregulação em processos infecciosos crônicos. Os relatos de interações idiotípicas em infecções parasitárias incluem estudos em várias doenças como malária, filariose, oncocercose, esquistosomose e doença de Chagas (revisado por COLLEY e cols., 1990). Estes estudos tratam basicamente da demonstração do papel de interações idiotípicas no sistema imune do hospedeiro e no desenvolvimento das alterações patológicas desencadeadas por essas infecções (HARTE & PLAYFAIR, 1983; SIDNER e cols., 1987; CESBRON e cols., 1988; ELOI-SANTOS e cols., 1989; SADIGURSKY e cols., 1988; GAZZINELLI, 1990). A identificação de idiotipos dominantes em anticorpos anti-parasita e sua correlação com estados clínicos da doença vem reforçar, através de uma abordagem molecular, a hipótese de que a expressão desses idiotipos tenha um papel de causa e/ou efeito na patogenia de tais infecções (OLDS & KRESINS, 1985; KRESINA & OLDS, 1986; MONTESANO e cols., 1989).

ESTUDO DO REPERTÓRIO IDIOTÍPICO EM PACIENTES CHAGÁSICOS CRÔNICOS

Provas da existência de interações idiotípicas na doença de Chagas crônica humana foram anteriormente relatadas por outros autores (SADIGURSKY e cols., 1988; GAZZINELLI e cols., 1988a; 1988b; 1990). Resultados destes estudos incentivaram a execução da primeira parte deste trabalho cuja proposta foi aprofundar no estudo do repertório idiotípico expresso nos anticorpos anti-epimastigota de *T. cruzi* (anti-EPI) imunopurificados do soro de pacientes chagásicos Cardíacos (IdC) e Indeterminados (IdI).

Enquanto GAZZINELLI e cols (1988a; 1988b; 1990) avaliaram a capacidade estimulatória destes idiotipos frente a células de pacientes chagásicos crônicos, utilizou-se neste trabalho uma abordagem molecular. A metodologia empregada baseia-se basicamente em medir a capacidade inibitória de preparações idiotípicas (IdC e IdI) em sistemas de ELISA-competição IdI / anti-IdI ou IdC / anti-IdC e ainda em avaliar a conformação destes idiotipos em "western blot". Analisaram-se os idiotipos expressos em anticorpos anti-EPI imunopurificados não apenas de "pools" de soros, mas também de amostras individuais de 10 pacientes Cardíacos e 8 Indeterminados.

Os resultados apresentados no **trabalho #1** mostram que, apesar dos idiotipos dominantes em anticorpos anti-EPI de pacientes chagásicos crônicos Cardíacos e Indeterminados não se diferirem quanto à sua reatividade anti-EPI ou ao seu perfil isotípico **(trabalho #1 - tabela 1, pag. 45),** eles são antigenicamente e estruturalmente diferentes. A capacidade apresentada por estas preparações idiotípicas de inibir as reações IdC / anti-IdC ou IdI / anti-IdI no sistema ELISA-competição tende a ser diretamente relacionada com a forma clínica do paciente da qual os anticorpos são purificados **(trabalho #1 - figs. 1 e 2, pag. 44)**. Os inibidores mais potentes

nos dois sistemas são as preparações idiotípicas das respectivas formas clínicas.

Em geral, o grau de inibição apresentado por IdC no sistema Indeterminado é bem maior quando comparado com a situação inversa. Em outras palavras, no sistema IdC / anti-IdC a concentração de IdI necessária para inibir 50% da reação é 4-8 vezes maior do que aquela requerida para IdC; enquanto no sistema IdI / anti-IdI, 50% de inibição da reação foi alcançado com uma concentração de IdC menor do que 2 vezes a requerida para IdI **(trabalho #1 - fig. 1, pag. 44).**

As preparações idiotípicas individuais apresentaram graus de inibição variáveis nos sistemas ELISA-competição Id /anti-Id, mas em geral elas se comportaram como os "pools" das respectivas formas clínicas. Dentre 10 casos Cardíacos analisados individualmente, dois apresentraram níveis de inibição no sistema IdC/anti-IdC comparáveis àqueles mostrados pelos Ids Indeterminados. **(trabalho #1 - fig. 3, pag. 45).** Esta variabilidade de expressão de IdC poderia estar relacionada com fases diferentes de progressão da cardiopatia chagásica.

A correlação de idiotipos dominantes em anticorpos anti-*T. cruzi* de pacientes chagásicos com diferentes formas clínicas da infecção crônica foi também demonstrada através de uma outra abordagem por GAZZINELLI e cols. (1988b). Estes autores relatam que anticorpos anti-*T. cruzi* purificados de soros de pacientes Cardíacos são significativamente mais eficientes na ativação de
linfócitos T do que aqueles purificados dos soros de pacientes Indeterminados.

No **trabalho #1** demonstra-se ainda que a expressão dos idiotipos dominantes em anticorpos anti-EPI imunopurificados de pacientes Cardíacos é quase completamente abolida através da redução das ligações di-sulfeto e consequente perda da conformação tridimensional da molécula de imunoglobulina. Por outro lado, apenas 10-30% dos idiotipos expressos em IdI são

destruidos pela redução da molécula destes anticorpos. **(trabalho 1 - figs. 4 e 5, pag. 46; tabela 2, pag. 46; figs. 6 e 7, pag. 47).** Com base nesses resultados pode-se inferir que a expressão dos idiotipos dominantes em IdC, ao contrário daqueles em IdI, é essencialmente dependente da conformação original da molécula de imunoglobulina. Esses dados são compatíveis com aqueles relatados por Gazzinelli e cols. (GAZZINELI e cols.,1990) mostrando que a estimulação de células mononucleares do sangue periférico (CMSP) de pacientes chagásicos crônicos não requer o processamento ou apresentação de IdC junto a antígenos de histocompatibilidades de classe II (MHC II). Ao contrário, a estimulação de CMSP de pacientes chagásicos por IdI requer o processamento e apresentação do idiotipo estimulador no contexto do complexo de antígenos de histocompatibildade classe II.

A análise molecular e estrutural destes idiotipos é sem dúvida um tópico de grande importância no estudo da cardiopatia chagásica crônica, tendo em vista a hipótese levantada por STEWART e cols. (1989) e VARELA e clos. (1991). Estes autores especulam que a expressão de certos polimorfismos genéticos da região hipervariável da moléula de imunoglobulina ou de receptores de células T (TCR) se refletiria na incapacidade do organismo em manter o equilíbrio do sistema de rede idiotípica e, consequentemente, desencadear um estado autoimune patológico. De fato tem sido observado que em certas doenças autoimunes, como artrite reumatóide e esclerose múltipla, existe uma certa tendência para a utilização de determinadas famílias gênicas da região variável de imunoglobulinas e/ou TCR (MARGUERIE e cols., 1992). Questões como estas tem sido abordadas molecularmente através de análises de recombinação dos segmentos gênicos dessas regiões variáveis. Isto tem sido realizado tanto utilizando marcadores sorológicos anti-idiotípicos associados a determinadas famílias gênicas, como a nível de sequenciamento de DNA.

Nesta tese pretendia-se caminhar para o estudo estrutural do idiotipo estimulador. Obtiveram-se 25 anticorpos monoclonais anti-*T. cruzi* a partir de camundongos cronicamente infectados

com este parasita e analisou-se a capacidade destes anticorpos estimularem CMSP de pacientes chagásicos. Utilizando o mesmo sistema de ELISA competitição Id / anti-Id relatado no trabalho #1, investigou-se ainda a semelhança antigênica entre IdC ou IdI e os idiotipos expressos nestes anticorpos monoclonais. Nos dois estudos acima os resultados foram negativos **(resultados não publicados)**. Esforços devem ser concentrados na produção de anticorpos monoclonais humanos.

Ressalta-se no **trabalho #1** a demonstração consistente de uma correlação da expressão de idiotipos dominantes com a forma clínica de pacientes chagásicos, o que vem reforçar a hipótese de que estes idiotipos teriam um papel de causa e/ou efeito na patogenia desta infecção crônica.

COLLEY e cols. (1990) especulam que IdC teria um papel direto na fase inicial do desenvolvimento da lesão cardíaca em pacientes portadores de doença de Chagas crônica. A sua expressão em moléculas de anticorpos ou de receptores de células T anti-*T. cruzi* estimularia intensamente células T anti-idiotípicas no coração. Estas células T anti-idiotípicas, por sua vez, liberariam uma variedade de linfocinas que agiriam diretamente causando danos nas células miocárdicas ou atraindo outras células inflamatórias para o local da lesão.

Dados da literatura sugerem que a fase crônica da doença de Chagas é marcada por alteração nos mecanismos imunoreguladores, com o consequente desequilíbrio das relações parasita / hospedeiro e ainda a perda do estado de tolerância pelo organismo. A expressão relativa de IdC e IdI pelo hospedeiro poderia influenciar tanto na fase inicial como na etapa final deste processo. É plausível imaginar que IdC, pela sua própria característica de estimular células T diretamente, sem requerer processamento ou apresentação junto a MHC II, poderia escapar ao sistema de conectividade da rede idiotípica (VAZ & FARIA, 1990) e influenciar diretamente, tanto o desequilíbrio parasita / hospedeiro, como a perda de tolerância pelo organismo. Assim se

desencadearia o estado patológico da fase crônica da infecção, o qual se manifesta de forma peculiar no coração.

ESTUDO IMUNOHISTOQUiMICO DA LESÃO CARDÍACA NA DOENÇA DE CHAGAS CRÔNICA

O estudo imunohistoquímico da lesão cardiaca é um tópico relevante na busca do entendimento da cardiopatia chagásica crônica, não só pelo fato desta constituir o ponto central desta doença, mas ainda por ser uma abordagem até então muito pouco utilizada em doença de Chagas humana. A maioria dos estudos nesta área foram realizados em animais de laboratório, principalmente camundongos.

YOUNES CHENNOUFI e cols. (1988) realizaram estudos histopatológicos de lesões em tecidos musculares cardíaco e esquelético de camundongos C3H e C57BL cronicamente infectados com a cepas CL e Y de *T. cruzi*. Estes autores mostraram que as lesões crônicas são constituidas predominantemente de neutrófilos (57%) e monócitos (25%). Os linfócitos representam cerca de apenas 6-12% das células inflamatórias totais, sendo que a maioria é CD4+ (Lyt 1+, Lyt 2-).

ROSSI (1990) observou que em camundongos Balb/c, após 40-60 dias de infecção com *T. cruzi* (cepa Y), o infiltrado inflamatório miocárdico é composto predominantemente de macrófagos expressando MHC II.

PIRMEZ e cols. (1990) analisaram a respoata imune de camundongos C57/BL trinta dias após infecção com a cepa Colombiana de *T. cruzi* e observaram infiltrados inflamatórios no miocárdio constituidos principalmente por macrófagos. De acordo com os autores, os linfócitos

compreendem cerca de 40% das células inflamatórias totais, sendo que destes, 60% são CD8+ e o restante é CD4+. Antígenos de histocompatibilidade de classe II foram detectados na superfície de alguns miocardiócitos.

SATO e cols. (1992) caracterizaram as sub-populações de linfócitos T em tecidos de ratos na fase aguda de infecção com *T. cruzi*. Estes autores mostraram que a infecção altera significativamente a relação linfócitos CD4+ / linfócitos CD8+ e a atividade citotóxica de células no baço, sangue e tecido miocárdico. Em todos estes compartimentos observou-se um predomínio de linfócitos CD8+ a partir do 9º dia de infecção até a morte que acontece em torno do 18º dia.

TARLETON e cols. (1992), estudando a susceptibilidade à infecção por *T. cruzi* apresentada por camundongos deficientes em $beta_2$-microglobulina, demonstraram a importância de linfócitos CD8+ na proteção a esta infecção. Este mesmo grupo de pesquisadores observou ainda que, nos tecidos, as células inflamatórias predominantes se tratam de linfócitos CD8+, tanto na fase aguda como na fase crônica da infecção (SUN & TARLETON, comunicação pessoal).

RIBEIRO DOS SANTOS e cols. (1992) estudaram o papel dos linfócitos CD4+ e CD8+ na cardiopatia da doença de Chagas experimental utilizando uma metodologia que consiste em transplantar corações de recém-nascidos nos lóbulos de orelhas de camundongos singeneicos. Estes autores mostraram que corações transplantados em camundongos cronicamente infectados são rejeitados, o que não é observado nos transplantes em animais normais. No entanto quando os animais infectados são pré-tratados com um anticorpo monoclonal anti-CD4, o fenômeno de rejeiçao é abolido. O mesmo não acontece se o pré-tratamento é feito com um anticorpo monoclonal anti-CD8. Além disto, a injeção de linfócitos CD4+ provenientes de animais infectados, no local do transplante em camundongos normais, é capaz de induzir o fenômeno de

rejeição. Finalmente, os autores comentam que cortes histológicos dos corações rejeitados mostram no local uma predominância de linfócitos CD4+.

Como pode ser observado pelo que foi descrito acima, os dados em doença de Chagas experimental são extremamente contraditórios. Diante disto e em vista da importância destas informações para a definição da natureza dos processos inflamatórios em curso no coração, resolveu-se desenvolver um estudo de caracterização imunohistoquímica da lesão cardíaca na doença de Chagas humana.

Em tecidos cardíacos provenientes de autópsia de cardiopatas chagásicos crônicos, demonstrou-se a presença constante de grandes focos inflamatórios e um intenso processo de fibrose. Esses focos inflamatórios são formados por uma grande maioria de de linfócitos T, cerca de 2-8% de linfócitos B CD20+, 1-8% de macrófagos CD68+ e 1-7% de células "natural killer" CD57+ **(trabalho #2 - tabela 3, pag. 60 e tabela 4, pag. 61)**. Cerca de 64-70% destas células expressam o marcador CD8 enquanto apenas 21-28% são CD4+ **(trabalho #2 - tabela 4, pag. 61 e figs. 1 e 2, pag. 62)**. O predomínio de linfócitos CD8+ na lesão chagásica crônica foi demonstrada também em estudos realizados concomitantemente por HIGUCHI (1991) e TOSTES JUNIOR (1993).

Com o objetivo de investigar o estado de ativação dos linfócitos T presentes na lesão chagásica crônica, estudou-se a expressão de 3 marcadores - CD45RO, HLA-DR e granzyme A. CD45RO é uma proteína encontrada em linfócitos T ativados ou "de memória" enquanto HLA-DR é a porção monomórfica do complexo de antígenos de histocompatibilidade de classe II que é naturalmente expressa em células B, macrófagos e células endoteliais, e é também um marcador de ativação celular em linfócitos T. Granzyme A é uma enzima, serina esterase, expressa por linfócitos T com atividade citotóxica (JENNE & TSCHOPP, 1988; GRIFFITHS & MILLER, 1991). Esta enzima é responsável por ativação de endonucleases com consequente

degradação de DNA de células alvo em processos de citotoxicidade (HAYES e cols., 1989).

A maioria (67-80%) dos linfócitos T presentes nestes focos inflamatórios expressam em sua superficie a proteína do grupo CD45RO **(trabalho #2 - tabela 3, pag. 60)** e a enzima granzyme A **(trabalho #2 - tabela 4, pag. 61 e fig. 3, pag. 62)** . Grânulos de granzyme A podem ser observados não apenas na superficie de linfócitos, como também no interstício e nos miocardiócitos. Apenas raros linfócitos e monócitos reagem com o anticorpo monoclonal específico para HLA-DR **(trabalho #3 - dados não mostrados).** No entanto, a expressão deste antígeno nas células endoteliais no coração de cardiopatas chagásicos crônicos é visualmente mais intensa do que a observada em individuos não chagásicos **(trabalho #3 - figs. 3 e 4, pag. 84)**.

Em resumo, a maioria das células inflamatórias na lesão cardíaca em pacientes portadores de doença de Chagas crônica é de de linfócitos T CD8+ que expressam a enzima granzyme A, sabidamente envolvida em processos de citotoxicidade. De 1- 7% destas células expressam o marcador de "natural killer" CD57 e apenas raras expressam CD56 ou CD16 (dados não mostrados). Observou-se ainda que as células miocárdicas desses mesmos indivíduos hiper expressam MHC I **(trabalho #3 - fig. 2, pag. 84 e tabela 2, pag. 81)** mas, ao contrário do que foi relatado por outros autores (ZANELLI e cols., 1990; PIRMEZ e cols., 1990), elas não expressam MHC II **(trabalho #3 - fig. 4, pag. 84)**. Nas células miocárdicas de indivíduos não chagásicos (n=7) **(trabalho #3 - fig. 1, pag. 84)** ou chagásicos assintomáticos (n=2) **(trabalho #3, tabela 2, pag. 81)**, detectou-se uma expressão de HLA-ABC muito baixa ou nula .

Estudos *in vitro* mostram que a expressão de MHC I por células miocárdicas pode ser estimulada por certas interleucinas usualmente liberadas em processos inflamatórios, principalemente TNF-alfa (WANG e cols., 1991). *In vivo*, o fenômeno de hiper expressão de MHC I já foi observado em casos de rejeição de transplantes (AHMED-ANSARI e cols., 1988;

ROSE e cols., 1986; DUSQUENOY e cols., 1987; STEINHOFF e cols, 1989; HUFNAGEL & MAISCH, 1991), em doenças viróticas (MAUDSLEY & POUND, 1991; SEKO e col.s, 1990) e ainda em outros processos inflamatórios (APPLEYARD e cols., 1985).

Na doença de Chagas não há dados suficientes para se definir o real significado da hiperexpressão de MHC I pelas células miocárdicas de indivíduos portadores de cardiopatia crônica. É possivel que os miocardiócitos sejam alvo de um processo de citotoxicidade restrito por MHC I, efetuado por linfócitos T CD8+ produtores de granzyme A. Neste caso, a degeneração miocárdica observada na cardiopatia chagásica crônica poderia ser explicada, em parte, por um processo de citotoxicidade, como aliás já foi sugerido por outros autores (SANTOS-BUCH & TEIXEIRA, 1974; TEIXEIRA e cols., 1978; RIBEIRO DOS SANTOS & HUDSON, 1980 ; MOLINA e cols., 1984; LAGUENS e cols., 1988).

Estudos anteriores demonstraram a atividade citotóxica *in vitro* de linfócitos de camundongos (RIBEIRO DOS SANTOS & HUDSON, 1980; LAGUENS e cols., 1988) e coelhos (SANTOS BUCH & TEIXEIRA, 1974) infectados com *T. cruzi* frente a células miocárdicas alogeneicas (SANTOS BUCH & TEIXEIRA, 1974, RIBEIRO DOS SANTOS & HUDSON, 1980) e singeneicas (LAGUENS e cols., 1988), parasitadas e não parasitadas. Resultados semelhantes foram obtidos por TEIXEIRA e cols. (1978) utilizando linfócitos obtidos de sangue periférico de pacientes chagásicos crônicos e agudos frente a miocardiócitos humanos. Todavia esta é a primeira vez que se demonstra a presença de linfócitos potencialmente citotóxicos diretamente na lesão chagásica cardíaca humana.

Não apenas linfócitos CD8+ ou mesmo CD4+, produtores de granzyme A, mas também macrófagos expressando TNF-alfa (tumor necrosis factor) **(trabalho #2 - tabela 4, pag. 83 e fig. 4, pag. 84),** parecem ter um papel no processo de citotoxicidade que ocorre na miocardite chagásica crônica. Resta investigar, neste caso, o envolvimento de outras interleucinas.

Os resultados aqui apresentados sugerem que um processo de citotoxicidade restrito por MHC I seja um dos mecanismos principais responsáveis pela destruição de miocardiócitos observada em indivíduos cardiopatas chagásicos. Além disto, na lesão miocárdica destes pacientes, foi encontrada também uma pequena porcentagem de células "natural killer", sabidamente efetoras de citotoxicidade não restrita por MHC. Outros relatos na literatura sugerem que processos de citotoxicidade mediada por anticorpos (MOLINA e cols., 1984) e citotoxicidade restrita por MHC II (ZANELLI e cols., 1990) também estejam envolvidos no processo de formação da lesão cardíaca da doença de Chagas humana. Os resultados dos **trabalhos #2 e #3** contradizem esta hipótese, pois não foi encontrado depósito de imunoglobulina e nem mesmo expressão de MHC II nas células miocárdicas. Estes estudos devem ser continuados através da pesquisa de outros marcadores, para que se defina melhor a contribuição relativa de cada um destes processos de citotoxicidade celular na formação da lesão miocárdica da doença de Chagas crônica humana.

A propósito, é importante lembrar que a pequena porcentagem de linfócitos CD4+, contrastante com a predominância de células CD8+ na lesão cardíaca da doença de Chagas crônica humana, não implica em um papel irrelevante dos linfócitos auxiliadores nos mecanismos efetores deste processo imunopatológico. Lesões tissulares, semelhantes àquelas da fase crônica, podem ser reproduzidas através da simples transferência de "pools" ou clones de células CD4+, de camundogos infectados para animais singeneicos não infectados (SAID e cols., 1985; YOUNNES-CHENNOUFI, 1988; RIBEIRO DOS SANTOS e cols., 1992). Ainda em camundongos, observou-se que células L3T4+ são capazes de produzir lesões decorrentes de reação de hipersensibilidade tardia específica a T. cruzi. (HONTEBEYRIE-JOSKOWICZ e cols., 1987). Deve-se proceder com cuidado ao extrapolar estes dados experimentais para a infecção humana, sem no entanto ignorá-los. Na doença de Chagas humana ainda está por se definir a contribuição relativa das sub-populações linfocitárias CD4+ e CD8+ na evolução da

cardiopatia crônica. A abordagem de estudo utilizada no **trabalho #2** não permite enfocar diretamente esta questão, mas algumas hipóteses podem ser levantadas e serão posteriormente apresentadas.

Com relação ao processo de fibrose, o parâmetro histopatológico da lesão que mais se relaciona com a severidade dos sintomas cardíacos, tais como cardiomegalia e insuficiência cardíaca congestiva (revisado por MORRIS e cols., 1990), um relato da literatura deve ser aqui citado para ilustrar a impotância de nossos achados. Em uma revisão sobre miocardite autoimune, ROSE e cols. (1991) chamam atenção para o fato de que camundongos não susceptíveis à infecção com o virus Coxsackie B, causador de miocardite autoimune experimental, podem se tornar susceptíveis e desenvolver a doença após o tratamento com TNF. No **trabalho #2** demonstramos a produção de TNF-alfa por macrófagos no local da lesão cardíaca em pacientes portadores de cardiopatia chagásica crônica. Estes macrófagos ativados presentes na lesão, expressando TNF-alfa e possivelmente outras linfocinas como Il-1 e fator de crescimento de fibroblastos, devem ter um papel central no processo de formação de fibrose cardíaca na cardiopatia chagásica crônica.

A última etapa desta tese inspira-se principalmente nos estudos de BARBOSA & ANDRADE (1984). Basicamente, o que estes autores mostraram foi que o *T. cruzi* pode ser encontrado nos mais diversos tecidos e órgãos de pacientes que morreram de cardiopatia chagásica crônica, com a ressalva de que apenas no coração se observa um processo inflamatório proeminente. Não se sabe a razão desta migração preferencial de células inflamatórias para o coração.

Comparando-se os dados relativos à razão linfócitos CD4+ / linfócitos CD8+, obtidos a partir de determinação fenotípica de células em coração e linfonodo **(trabalho #2)** e ainda em sangue periférico (ORNELAS, comunicação pessoal) de pacientes chagásicos Cardíacos, encontram-se as seguintes médias: para o coração = 0,36; para o linfonodo = 1,8 ; e para o sangue

periférico=1,7. A análise destes dados não apenas confirma como também complementa os estudos de BARBOSA & ANDRADE (1984). Em indivíduos cardiopatas chagásicos, as células inflamatórias que migram preferencialmente para o coração tratam-se principalmente de linfócitos T CD8+.

Em um processo inflamatório o padrão de migração dos leucócitos é definido por uma série de interações a que estas células se submetem. Estas interações ocorrem não somente através de respostas específicas a antígenos externos, como também via rede idiotípica. Paralelamente são estabelecidas interações entre moléculas expressas nas superfícies de leucócitos e seus ligantes em outras células ou na matriz extracelular. Tais moléculas são genericamente denominadas "moléculas de adesão". Devem-se adicionar aqui também as interleucinas, que não só exercem um efeito direto como fatores quimiotáticos, mas também indireto através da indução de expressão de moléculas de adesão.

Estudou-se a expressão de moléculas de adesão em tecidos cardíacos de pacientes chagásicos e

indivíduos não chagásicos e os resultados se encontram relatados no **trabalho #3.** Nas células endoteliais investigou-se a expressão de ELAM (endothelial leucocyte adhesion molecule) e ICAM-1 (intercellular adhesion molecule). Observou-se que ICAM-1 é expressa no endotélio dos vasos e capilares, indistintamente em pacientes chagásicos e em indivíduos não chagásicos **(trabalho #3 - tabela 4, pag. 83).** ELAM é sintetizada pelas células endoteliais especialmente nos vasos localizados próximos a focos inflamatórios e apenas nos casos de cardiopatia chagásica crônica **(trabalho #3 - tabela 4, pag. 83 e figs. 5 e 6, pag. 85)**. Convém lembrar que, apesar de ELAM ser considerada uma molécula de adesão de processos inflamatórios agudos, estudos *in vitro* demonstram que sua síntese nas células endoteliais pode ser induzida por algumas interleucinas especialmente TNF-alfa e IL-1. Estas observações são condizentes com nossos resultados mostrando a expressão de ELAM pelas células endoteliais e ainda de TNF-

Nos linfócitos foram estudadas as seguintes moléculas de adesão: LFA-1 (leucocyte function antigen), LFA-3, VLA-4 (very late antigen) e CD44 (leucocyte homing receptor). LFA-1 é o receptor para ICAM-1 e LFA-3 se liga a CD2 (PARDI e cols., 1992). Tanto VLA-4 e CD44 ligam-se a fibronectina, sendo que o primeiro é ainda receptor para VCAM (vascular adhesion molecule) e o segundo para ácido hialurônico e colágeno (CULTY e cols., 1990; ARUFFO e cols., 1990; DUSTIN & SPRINGER, 1991; PARDI e cols., 1992). VLA-4, CD44 e LFA-1 são intensamente expressas por células citotóxicas em casos de rejeição de transplantes em camundongos (MOBLEY & DAILEY, 1992).

A maioria dos linfócitos na lesão chagásica cardíaca expressam LFA-1 **(fig. 7, pag. 85)**, CD44 **(fig. 8, pag. 85)** e VLA-4 **(fig. 9, pag. 85)** e raras destas células apresentam LFA3 fracamente em sua superfiicie (dados não mostrados) **(trabalho #3)**. A expressão de VLA-4, CD44 e LFA-1 pela maioria dos linfócitos na miocardite chagásica crônica deve ter alguma influência no processo inflamatório, não apenas mediando a migração seletiva destas células ao coração, como também promovendo sua adesão ao endotélio e auxiliando no seu tráfico através da matriz extracelular.

Em resumo, no coração de um indivíduo portador de cardiopatia chagásica crônica observam-se: 64-70% de linfócitos CD8+, 21-28% de linfócitos CD4+, cerca de 2-8% de linfócitos B, 1-7% de células "natural killer" e 1-8% de macrófagos produtores de TNF alfa. As células miocárdicas expressam intensamente MHC I e a maioria dos linfócitos são produtores de granzyme A. Na superficie das células endoteliais observa-se uma expressão aumentada de MHC II e ELAM. A maioria dos linfócitos expressam LFA-1, CD44 e VLA-4 e raros expressam LFA-3.

Os resultados apresentados nesta tese são informações inéditas, de grande relevância para a compreensão da miocardite da forma crônica da doença de Chagas humana. Embora a metodologia de estudo aqui utilizada não permita abordar diretamente os mecanismos patogênicos desta miocardite, as informações obtidas podem ser utilizadas na montagem de um cenário especulativo a cerca deste processo.

Estudos recentes mostram que o *T. cruzi*, apesar de escasso, está sempre presente na lesão miocárdica (JONES e cols., 1993).É possível que, na fase inicial deste processo inflamatório, predomine no coração uma reação de hipersensibilidade tardia desencadeada por linfócitos T CD4+ contra antígenos do parasita alojados no local. Vários fatores poderiam contribuir para o posterior recrutamento de linfócitos CD8+ para o coração: interleucinas secretadas por linfócitos CD4+, a expressão de MHC I pelas células miocárdicas e ainda a expressão de moléculas de adesão pelo endotélio dos vasos locais.

Também as células endoteliais, que expressam intensamente HLA-DR, parecem ser componentes ativos do processo inflamatório em curso no coração de pacientes portadores de cardiopatia chagásica crônica. Estudos *in vitro* apoiam esta hipótese ao demonstrar que células endoteliais, se estimuladas por interferon-gama, expressam intensamente HLA-DR e são capazes de apresentar antígenos e induzir eficientemente a estimulação de linfócitos T (HIRSCHBERG e cols., 1975; WAGNER e cols., 1984)). Antígenos de *T. cruzi*, que sabidamente infectam células endoteliais humanas *in vivo* (TANOWITZ e cols., 1990), poderiam ser processados e apresentados junto a MHC II para os linfócitos CD4+ presentes no coração.

O *T. cruzi* é um parasita que se replica no interior das células infectadas e, por isso, é de se esperar que alguns de seus epitopos possam ser expressos também no contexto de MHC I. JONES e cols (1993) demonstraram a presença de fragmentos de DNA de *T. cruzi* no coração de 100% de 7 chagásicos cardiopatas analisados. Estes autores discutem a localização destes

fragmentos de DNA e levantam a possibilidade deles se encontrarem integrados ao genoma do hospedeiro, como foi também sugerido por TEIXEIRA cols. (1991). Neste caso, as células miocárdicas poderiam passar a expressar peptídeos do *T. cruzi* e apresentá-los junto a MHC I aos linfócitos citotóxicos CD8+.

Apesar do *T. cruzi* estar sempre presente no coração de cardiopatas chagásicos, a sua escassez contrastante com a intensidade do processo inflamatório, sugere a existência de uma reação secundária contra antígenos do próprio hospedeiro. Esta reação autoimune seria parcialmente responsável pela perpetuação do processo inflamatório e agravamento da lesão miocárdica. Neste sentido é interessante observar que, o cenário da lesão chagásica cardíaca aqui descrito se assemelha muito à configuração do processo imunopatológico em casos de rejeição de transplantes. Em ambos os casos observa-se no local da lesão uma predominância de linfócitos CD8+, uma escassez de linfócitos B (**trabalho #1**; HIGUCHI e cols., 1991), células inflamatórias expressando TNF (**trabalho #1**, ARBUSTINI e cols., 1991) ou granzyme A (**trabalho #1**, GRIFFITHS e cols., 1991) e expressão de MHC I por miocardiócitos (**trabalho #2**, AHMED-ANSARI e cols., 1988; ROSE e cols., 1986; DUSQUENOY e cols., 1987; STEINHOFF e cols., 1989; HUFNAGEL & MAISCH, 1991). .

É provável que a existência de lesões miocárdicas intensas possa ser justificada pela somatória de vários mecanismos de destruição celular: citólise causada por parasitismo, ação direta de interleucinas, citotoxicidade celular mediada por anticorpos, por células "natural killer"ou ainda por linfócitos CD4+. No entanto, o mecanismo predominate na lesão miocárdica crônica humana parece ser a citotoxicidade mediada por linfócitos CD8+, provavelmente via MHC I. Por isto, é preciso que estas células sejam caracterizadas quanto a sua especificidade, síntese de interleucinas e utilização de receptores de células T.

RESUMO E CONCLUSÕES

Realizamos este estudo cientes da importância do modelo humano no estudo da doença de Chagas crônica e tentando ultrapassar as limitações que este modelo oferece. Estudamos o repertório idiotípico de pacientes chagásicos Cardíacos e Indeterminados e descrevemos algumas características da lesão inflamatória no coração, sempre visando compreender o desenvolvimento da cardiopatia da doença de Chagas crônica humana.

Demonstramos que o padrão de expressão idiotípica em anticorpos anti-EPI imunopurificados de soro ou plasma de pacientes chagásicos cardiopatas (IdC) difere daquele expresso em imunoglobulinas de portadores da forma clínica Indeterminada (IdI). A expressão dos idiotipos dominantes em IdC, ao contrário daqueles encontrados em IdI, é dependente da conformação original da molécula de imunoglobulina. Esta correlação encontrada entre padrão de expressão idiotípica e forma clínica sugere um papel de causa e/ou efeito destes idiotipos na patogenia da cardiopatia chagásica crônica.

Mostramos que os infiltrados inflamatórios presentes no miocárdio de indivíduos que morreram de cardiopatia chagásica crônica são constituidos de cerca de 64-87% de linfócitos T, 2-8% de linfócitos B CD20+, 1-7% de células "natural killer" CD57+ e 1-8% de macrófagos CD68+. A grande maioria dos linfócitos T expressa CD45RO, marcador de células ativadas e/ou de memória.

Observamos ainda que os linfócitos T CD8+ e CD4+ constituem 64-70% e 21-28% respectivamente das células inflamatórias totais na lesão miocárdica, sendo que a relação CD4 / CD8 encontrada foi de $0{,}36 \pm 0{,}03$. Cerca de 38-65% das células inflamatórias expressam granzyme A, enzima envolvida em processos de citotoxicidade. Além disto, demonstramos que nos miocardiócitos de chagásicos cardiopatas a expressão de MHC I é muito mais intensa do

que aquela observada em chagásicos assintomáticos ou não chagásicos. Estes dados nos levam a sugerir que o principal mecanismo responsável pela destruição de miocardiócitos na cardiopatia chagásica crônica humana seja a citotoxicidade mediada por linfócitos T CD8+ e restrita por MHC I.

Finalmente estudamos a expressão de moléculas de adesão pelos leucócitos e células endoteliais no coração de pacientes chagásicos e indivíduos não chagásicos. Observamos que a maioria dos leucócitos na lesão chagásica cardíaca expressam CD44, VLA-4 e LFA-1, As células endoteliais nos corações de chagásicos cardiopatas expressam ELAM, sintetizam intensamente HLA-DR e devem, por isto, participar efetivamente do processo inflamatório da cardiopatia da doença de Chagas crônica humana.

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Antibodies to *Trypanosoma cruzi* Express Idiotypic Patterns that Can Differentiate between Patients with Asymptomatic or Severe Chagas' Disease[1]

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ABSTRACT. Immunization of rabbits with pools of immunoaffinity-purified anti-*Trypanosoma cruzi* epimastigote antibodies derived from patients with different clinical forms of Chagas' disease induces antiidiotypic sera that can distinguish between anti-epimastigote antibodies from patients with asymptomatic (indeterminate (IND)) or severe (cardiac (CARD)) Chagas' disease. These idiotypically different anti-EPI antibodies from patients with the different clinical forms do not differ in their antiepimastigote activities or isotypes. Analysis of immunoaffinity purified antibodies from individual chagasic patients by specific competitive ELISA generally confirms that Id-specific rabbit antisera can differentiate the clinical forms of the source of the antibodies. Based on these data, immunoaffinity-purified antibodies from patients share many Id with those from IND patients, although antibodies from IND patients express much lower levels of the distinctive Id characteristic of CARD patients. Reduction and alkylation of antibodies from IND patients reduces somewhat, but does not abolish, the ability of their Id to be recognized idiotypically, and to effectively inhibit in competitive ELISA. In contrast, reduction and alkylation of antibodies from CARD patients almost completely eliminates the ability of their predominant Id to be either recognized by, or inhibit, the appropriate systems. These data imply that the expression of the major Id that define CARD patients by these serologic anti-Id systems is largely dependent on the tertiary conformation of the Ig molecule. This agrees with our earlier studies on the respective differential abilities of CARD vs IND Id to stimulate anti-Id T cells by direct stimulation vs processing and presentation mechanisms. *Journal of Immunology,* 1993, 150: 1611.

Chagas' disease is a predominantly rural disease affecting millions of people in Latin American countries. Acute Chagas' disease is characterized by fever and myocarditis related to intracellular parasitism by the causitive protozoan, *Trypanosoma cruzi*. Usually these symptoms subside spontaneously. Most patients then remain serologically positive, but asymptomatic, for the rest of their lives, with normal physical, radiologic, and electrocardiographic characteristics. Such patients are termed IND[4] cases (1). Unfortunately, some patients

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[4] Abbreviations used in this paper: IND, indeterminate; CARD, cardiac; EPI, epimastigote; NHu, normal human.

progress beyond the chronic IND stage, and after a prolonged asymptomatic infection, develop chronic, active, fibrosing myocarditis (2) and are classified as CARD patients based on electrocardiographic and chest x-ray data (1). These opposite ends of the clinical spectrum of chronic Chagas' disease both occur in the relative absence of routinely demonstrable parasites in either the blood or tissues (3).

The pathogenic mechanisms responsible for the IND to CARD transition are not understood. Both direct induction by a few parasites, and autoimmune phenomena have been proposed to participate in this progression (4–7). There is a suggestion that polyclonal activation of the immune system during the acute infection could liberate anti-self clones that would persist for long periods in the host (8), and there are multiple examples of the presence of Ag that are shared or cross-reactive between *T. cruzi* and host heart and neuronal tissue (9–13).

In earlier studies, we demonstrated that anti-Id T cells develop in chronic Chagas' patients, and these cells proliferate differentially in vitro to anti-EPI antibodies immunoaffinity-purified from the sera of patients with either the IND or CARD forms of Chagas' disease (14, 15). The Id expressed by anti-EPI antibodies purified from the sera of chronic CARD patients (IdC) were inherently more stimulatory than those anti-EPI antibodies from sera of chronic IND patients (IdI) for T cells of patients with either IND or CARD disease. Recently, a difference in the recognition requirements for chronic chagasic anti-Id T cell responses to IdC compared to IdI became evident (16, 17). We reported that in Chagas' disease anti-Id T cell stimulation can proceed via two distinct mechanisms. Stimulation of T cells from IND patients requires processing and presentation of the Id expressed on anti-EPI antibodies, regardless of whether the Id were prepared from IND or CARD patients. In contrast, stimulation of T cells from CARD patients requires processing and presentation only if the Id is from IND patients (IdI), but not if the Id is from CARD patients (IdC). The latter system (CARD anti-Id T cells plus IdC) proceeds through an undefined direct stimulation pathway (16).

Our study further defines differences in the Id expressed on the anti-EPI antibodies of IND vs CARD chagasic patients by using anti-Id serology. In previous studies, Western blot analyses showed that immunoaffinity-purified anti-EPI antibodies from these two clinical sources do not differ in their general recognition patterns of different EPI antigens (14). We now show that when these antibody preparations are made from either pooled or individual sera from IND vs CARD patients they do, however, differ in their abilities to react with, and compete in, various Id-specific serologic assays. Furthermore, these serologic data imply that in contrast to the predominant Id from IND patients, the dominant Id from most CARD patients are largely dependent on the tertiary conformation of the Ig molecules, as was predicted based on their direct recognition by anti-Id T cells (18).

Materials and Methods

Patients

All chronic chagasic patients in this study came from endemic areas in the state of Minas Gerais, Brazil. They were categorized as having either chronic IND or CARD Chagas' disease based on physical examination, chest x-ray, and electrocardiography. Sera from all of these patients were positive in complement fixation and indirect immunofluorescence assays for Chagas' disease. Informed consent was obtained from all patients before their inclusion in the study.

Anti-EPI antibodies from CARD and IND chagasic patients

The soluble EPI antigenic preparations used in this study were obtained as described previously (19). Pooled anti-EPI antibodies were immunoaffinity purified from pooled sera of 17 or 12 CARD (IdC), or 11 or 12 IND (IdI) patients. Individual anti-EPI antibodies were immunoaffinity purified from the individual sera from patients with either IND or CARD infections (10 CARD, 8 IND). Immunoaffinity purifications were done similarly to those reported previously (14–17). Sera were passed over an EPI-Sepharose 4B (Sigma Chemical Co., St. Louis, MO) FPLC column (HR 10/10; Pharmacia Fine Chemicals, Piscataway, NJ), and the elution of the bound antibodies was done with 0.1 M glycine-HCl, pH 2.8, at a flow rate of 1.4 ml/min. Purified antibodies were neutralized, vacumn concentrated, and their protein content measured (Pierce BCA Protein Assay Method; Pierce Chemical Co., Rockford, IL). Based on silver-stained samples subjected to 10% SDS-PAGE analysis, these preparations approximated 100% Ig (data not shown).

Preparation of anti-Id antisera

Rabbits were immunized with 100 μg of pooled anti-EPI antibodies (IdC or IdI). The Ig preparations were first administered in CFA, and each rabbit was boosted 2 and 4 wk after primary injection with 150 μg of the respective Ig pool in IFA. Antisera were obtained 2 wk after the last injection. Each antiserum was precipitated at 45% $(NH_4)_2SO_4$, dialyzed against PBS, and absorbed 10 times with 20 ml Sepharose 4B (Sigma) conjugated with 10 mg of NHuIg (Sigma). The final unbound preparations were termed anti-IdI or anti-IdC, depending on the source (IND or CARD patients) of the original immunizing anti-EPI

antibodies. Aliquots of anti-IdI and anti-IdC were biotinylated by adding NHS-LS-biotin (200 μg) (Sigma) to 10 mg of anti-IdI or anti-IdC, and incubating for 2 h on ice. The solutions were dialyzed against PBS, aliquoted and stored (−20°C).

Anti-EPI ELISA

EPI solutions (100 μl) at 10 μg/ml in 0.1 M carbonate buffer, pH 9.5, were put in wells of flat-bottom Immulon 2 microtiter plates (Dynatech Laboratories, Inc, Alexandria, VA) at 4°C, overnight. Plates were washed three times with PBS containing 0.05% Tween 20, and uncoated plastic sites were blocked by 200 μl of 2% BSA in PBS for 2 h, at room temperature. Washed plates were incubated with serial dilutions of pooled or individual anti-EPI immunoaffinity-purified antibodies (IdI or IdC) in PBS-Tween for 1 h, at room temperature, and antibody binding to EPI detected by rabbit anti-human Ig-biotin conjugates (Sigma) (1 h), extravidin-peroxidase conjugate (Sigma) (30 min), and addition of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) (10 min). The reactions were stopped with 25 μl of 10% SDS, and the absorbances measured on a microplate reader (Dynatech Instruments, Inc., Torrance, CA).

Quantification of total IgG and IgM in anti-EPI samples

Microtiter ELISA plates were sensitized with 20 μg/ml of polyclonal rabbit anti-human Ig (Sigma) in 0.1 M carbonate buffer, pH 9.5, at 4°C, overnight. Plates were then washed three times with PBS/Tween and uncoated plastic sites were blocked by 200 μl of 2% BSA in PBS at room temperature for 2 h. Id samples (5, 10, and 20 μg/ml) diluted in PBS/Tween were added to the wells and further incubated for 1 h at room temperature. Human IgG and IgM (Sigma) were used to construct standard curves. Bound IgG or IgM was revealed using biotin labeled goat anti-human γ- or μ-chains and extravidin-peroxidase conjugate. ELISA plates were developed as described above. The concentration of IgG or IgM in each sample was determined by extrapolation from the standard curves.

Reduction and S-carboxymethylation of Ig

Id preparations and NHuIg were reduced and S-carboxymethylated. Each sample (500 μg) was diluted in 6 M guanidinium hydrochloride (2.5 ml) and dithiothreitol added to a final concentration of 10 mM. Samples were incubated at 37°C for 2 h and iodoacetate added to a final concentration of 10 mM. Samples were then incubated at 0 to 4°C for 3 h and extensively dialyzed against PBS.

ELISA for detection of anti-Id activity

ELISA assays for detection of anti-Id activity in purified, biotinylated antibodies from anti-EPI immunized rabbits were essentially done as described above for measuring anti-EPI activity. Pooled anti-EPI antibodies (IdC and IdI) or NHuIg (Sigma) were adsorbed on Immulon 2 microplates (3 μg/ml, 100 μl). The ability of the different anti-anti-EPI antibodies (anti-IdC and anti-IdI) to react with complementary antibodies, but not NHuIg was detected by incubating the Id-coated plates with serial dilutions of anti-IdI or anti-IdC antibodies.

Competitive ELISA

The complementary systems (IdC/anti-IdC and IdI/anti-IdI) were used to construct Id/anti-Id competitive ELISA. Anti-IdC or anti-IdI was preincubated (2 h) at room temperature with increasing concentrations (0.32–15.0 μg/ml) of complementary Id preparations (anti-IdC/IdC and anti-IdI/IdI), noncomplementary pooled Id preparations (anti-IdC/IdI and anti-IdI/IdC), individual Id preparations (IdC1-IdC10 and IdI1-IdI8), or NHuIg, all in the reduced or nonreduced form. The incubation mixtures were then added to IdC or IdI coated-plates and evaluated for the binding of the biotinylated complementary system, as described above. The percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = 1 - \frac{\text{OD in presence of inhibitor} - \text{control OD}}{\text{OD in absence of inhibitor} - \text{control OD}} \times 100$$

A percent inhibition curve was generated in each experiment using a concentration curve for each potentially inhibitory Id. The concentration of each Id preparation needed to achieve a 50% level of inhibition in relationship to the complementary system was calculated by linear regression analysis.

Western blot

SDS-PAGE was performed under reduced and nonreduced conditions. Samples (5 μg/lane of NHIg, IdCp1, or IdIp1) in 2% SDS and 10% glycerol, with or without 2% of β-mercaptoethanol, were electrophoresed in a 10% gel for 1 h, using the Mini Electrophoresis System (Bio-Rad Laboratories, Richmond, CA). After electrophoresis, electroblotting was done with nitrocellulose sandwiches. The immunoblotted membranes were blocked for 2 h in PBS with 0.05% Tween-20 and 3% nonfat dry milk. The nitrocellulose strips were then incubated for 1 h with 50 μg/ml of biotin-labeled anti-IdIp1 or anti-IdCp1, followed by a 1 h incubation with an avidin-peroxidase conjugate. Each incubation was at room temperature with shaking and between incubations the strips were washed five times, 5 min

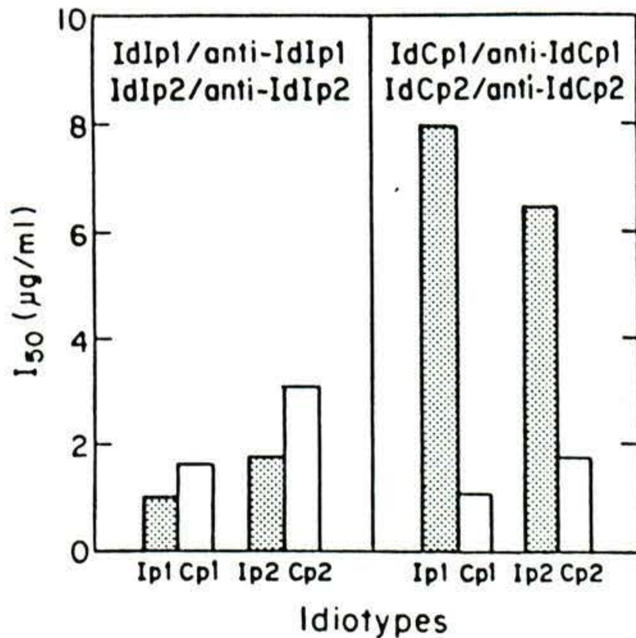

FIGURE 1. Competitive ELISA assays. The pooled Id (Ip1, Ip2, Cp1, and Cp2) (3 μg/ml) were used to coat ELISA plates. The inhibitory abilities of each pooled Id in complementary or in noncomplementary Id/anti-Id systems were evaluated by incubating each one with biotin-labeled anti-Id preparations at dilutions of 1/450. Percent inhibition curves were generated of each inhibitor and an 50% level of inhibition (I_{50}) was calculated for each, as described in the text. The results in the *left panel* are the inhibitions in two different indeterminate systems: the *left-hand pair* in the IdIp1/anti-IdIp1 system (I_{50} of Id Ip1 ☐ and Cp1 ☐) and the *right-hand pair* in the IdIp2/anti-IdIp2 system (I_{50} of Id Ip2 ☐ and Cp2 ☐). The *right panel* shows the results with two different cardiac systems: the *right-hand pair* in the IdCp1/anti-IdCp1 system (I_{50} of idiotypes Ip1 ☐ and Cp1 ☐) and the *right-hand pair* in the IdCp2/anti-IdCp2 (I_{50} of idiotypes Ip2 ☐ and Cp2 ☐).

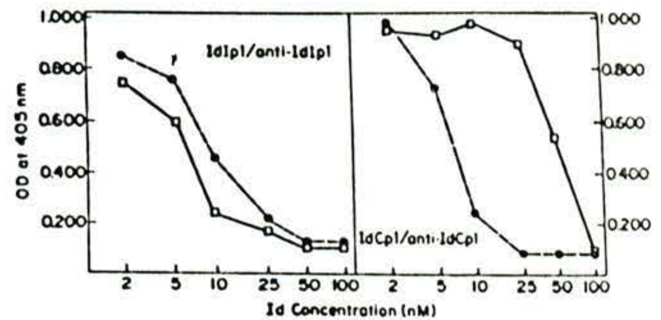

FIGURE 2. Competitive ELISA: titration of the inhibitory abilities of IdIp1 (☐) and IdCp1 (●) in the respective complementary and noncomplementary systems. Biotin-labeled anti-IdIp1 and anti-IdCp1 were preincubated with different concentrations of IdIp1 or IdCp1 and the mixtures were added to, respectively, IdIp1- or IdCp1-coated plates. The concentrations (nM) of each inhibitor in the anti-Id solutions were calculated considering the molecular mass of IgG molecule as 150 kDa.

each, with PBS-Tween 0.05%. Two NHIg strips were incubated with goat anti-human Ig-peroxidase conjugate (Sigma) as reduced or nonreduced Ig controls. The reactions were developed with 3-3'-diaminobenzidibe and 4-chloro-1-naphthol.

Results

Several specific rabbit anti-Id reagents demonstrate distinct and shared Id expressed on anti-EPI antibodies from IND and CARD patients

Rabbits were immunized with pooled anti-EPI antibodies from two different groups of IND patients (IdIp1 or IdIp2) or two different groups of CARD patients (IdCp1 or IdCp2). After purification, NHuIg absorptions and biotinylation, the anti-Id activities of these antisera were determined by homologous (complementary) (IdIp1/anti-IdIp1, IdIp2/anti-IdIp2, IdCp1/anti-IdCp1, or IdCp2/anti-IdCp2) and anti-NHuIg ELISA assays. The optimum point of complementary Id/anti-Id-specific reaction was determined for each reagent (data not shown). Dilutions of pooled Id were assayed for their ability to inhibit the IdIp1/anti-IdIp1, IdIp2/anti-IdIp2, IdCp1/anti-IdCp1, and IdCp2/anti-IdCp2 ELISA systems. The incubation of each anti-Id with increasing concentrations of its corresponding, complementary Id always led to complete (100%) inhibition of the reaction (data not shown). Competitive ELISA assays demonstrated some level of serologic homology among all anti-EPI antibodies from both CARD and IND patients, showing that each was able to compete, albeit at different levels of efficiency, in each system (Fig. 1). However, in each case, the corresponding, complementary pooled Id was the best competitor, i.e., they required lesser amounts to achieve an I_{50}. Furthermore, the ratios needed differed greatly depending on whether one tried to inhibit an IND complementary system with CARD Id or a CARD complementary system with an IND Id. Thus the ratios between the amount of each IdC and its counterpart IdIp to inhibit 50% of the reaction in IdIp/anti-IdIp systems were minimal (1.6 for IdIp1/anti-IdIp1 and 1.7 for IdIp2/anti-IdIp2), whereas much higher ratios were required to inhibit in the opposite direction (7.9 for IdIp1 in IdCp1/anti-IdCp1 and 3.9 for IdIp2 in IdCp2/anti-IdCp2, respectively). In other words, in an IdC/anti-IdC system it required four- to eightfold as much IdIp as IdCp to reach the level of 50% of inhibition, whereas less than twofold as much IdCp as IdIp was needed to achieve 50% inhibition of an IdI/anti-IdI competitive ELISA. This suggests that anti-EPI antibodies from CARD patients share many Id expressed on anti-EPI antibodies from IND patients, although anti-EPI antibodies from IND patients express considerably fewer of the Id that are characteristics of CARD anti-EPI antibodies. An individual titration of this situation using IdIp1 and IdCp1 is presented in Figure 2.

Table I
Characterization of individual cardiac and indeterminate idiotypic preparations

Idiotypic Preparation	Anti-EPI Reactivity[a]		Isotype Characterization[b]	
	Id dilution (μg/ml)		% IgG	% IgM
	2.500	0.156		
IdI1	0.961	0.304	75	10
IdI2	0.644	0.276	90	3
IdI3	0.607	0.243	86	3
IdI4	0.706	0.316	73	26
IdI5	0.787	0.390	91	2
IdI7	0.674	0.313	89	7
IdI8	0.715	0.323	98	2
IdC1	0.787	0.304	88	3
IdC2	0.702	0.377	82	8
IdC3	0.975	0.366	97	2
IdC4	0.686	0.400	88	11
IdC5	0.618	0.332	98	1
IdC6	0.755	0.337	95	3
IdC7	0.654	0.307	92	4
IdC8	0.681	0.302	91	3
IdC9	0.748	0.317	89	5
IdC10	0.648	0.316	90	6

[a] ELISA plates were coated with EPI (10 μg/ml), washed, blocked and incubated with individual Id preparations from chagasic patients of the cardiac (C1-C10) and indeterminate (I1-I8) clinical forms. Bound anti-EPI activity was detected by using goat anti-human biotinylated antibodies, extravidin-peroxidase, and ABTS, and the values are given as OD at 405 nm. Replicate studies consistently yielded comparable data.

[b] Id preparations were incubated on rabbit anti-human Ig-coated plates and developed with goat anti-human IgG or goat anti-human IgM. Human IgG and human IgM (Sigma) were used to generate standard curves, the concentration of IgG or IgM in each sample determind based on those curves and the percent of each calculated.

Idiotypic profiles of anti-EPI antibody preparations from individual IND and CARD patients

To further study the distribution of the Id expressed on anti-EPI antibodies purified from Chagas' patients with either the IND or CARD clinical forms of infection, anti-EPI antibodies were purified from the sera of individual patients rather than from sera first pooled (as above) on the basis of the patients' clinical form. These individual preparations (10 of CARD; 8 of IND) were of the same purity as the pooled Id, based on silver staining after 10% SDS-PAGE, and their overall anti-EPI reactivities and isotype patterns did not differ significantly (Table I).

IdIp2/anti-IdIp2 and IdCp2/anti-IdCp2 ELISA assays were used to analyze the inhibitory ability of these individual Id (Fig. 3). In 8 of 10 persons tested with cardiac disease the idiotypic analysis was clearly different than in 8 of 8 persons tested with indeterminate form of the infection. All eight of the individual IdI preparations were highly comparable in their abilities to inhibit the IdI/anti-IdI system. On the average, more individual IdC was needed to achieve I_{50} levels in the IdI/anti-IdI system, but 2 of 10 of these CARD preparations were as efficient as IND preparations. The mean I_{50} concentrations in the IND system were 2.04 ± 0.30 μg/ml for IND Id and 3.53 ± 0.99 μg/ml for CARD Id (these means are significantly different at the 0.05 level). These values were very similar

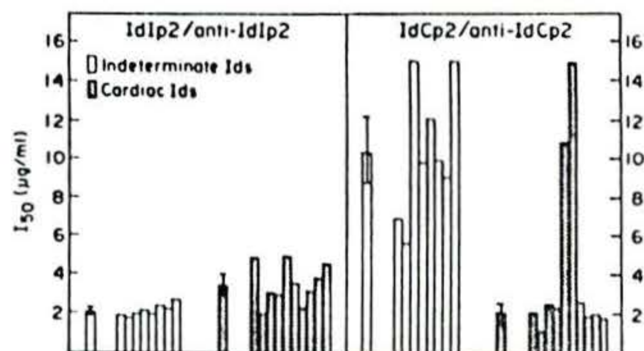

FIGURE 3. Competitive ELISA assay was used to evaluate the ability of indeterminate individual Id (1–8, from *right* to *left*) and cardiac individual Id (1–10, from *right* to *left*) to inhibit binding in IdIp2/anti-IdIp2 and IdCp2/anti-IdCp2 systems. Biotin-labeled anti-IdIp2 anti-IdCp2 were preincubated with increasing concentrations of Id (0.32–0.15 μg/ml) and placed in, respectively, IdIp2 or IdCp2-coated ELISA plates. A 50% level of inhibition (I_{50}) was calculated for each set as described in the text. The mean I_{50} ± SD concentrations for each group are given in the *bars* with SD brachets. The mean value for individual IdC samples does not include the data from outlier patients 5 or 6.

to what was observed with the competitor pooled Id (Fig. 1).

Figure 3 also presents the data from the reverse situation, where these individual Id preparations were used to inhibit the IdC/anti-IdC system. As with the pooled Id systems (Figs. 1 and 2), these assays usually present the reverse picture of the IdI/anti-IdI system, and further indicate that most CARD patients have anti-EPI Id profiles that are very similar to those of the pooled IdC. This is clearly not true for the Id profile expressed by the antibodies from all CARD patients, in that CARD patients 5 and 6 did not express high levels of what we term IdC, but rather express levels (I_{50} concentrations of 11.5 and 15.0 μg/ml) normally associated with IND patients. The clinical data on these two patients did not differ by any known parameters from the other cardiac patients and their "outlier" status remains unexplained.

Reduction of antibodies defines distinct Id populations expressed by antibodies from either IND or CARD patients

Western blots using IdIp1/anti-IdIp1 and IdCp1/anti-IdCp1 systems were used for preliminary investigations of the conformational nature of Id expressed on anti-EPI antibodies from CARD and IND chagasic patients. Figure 4 shows that anti-IdIp1 bound to both IdIp1 and IdCp1 under reduced and nonreduced conditions. In contrast, whereas anti-IdCp1 bound to both IdCp1 and IdIp1 under nonreduced conditions, it did not bind either idiotypic

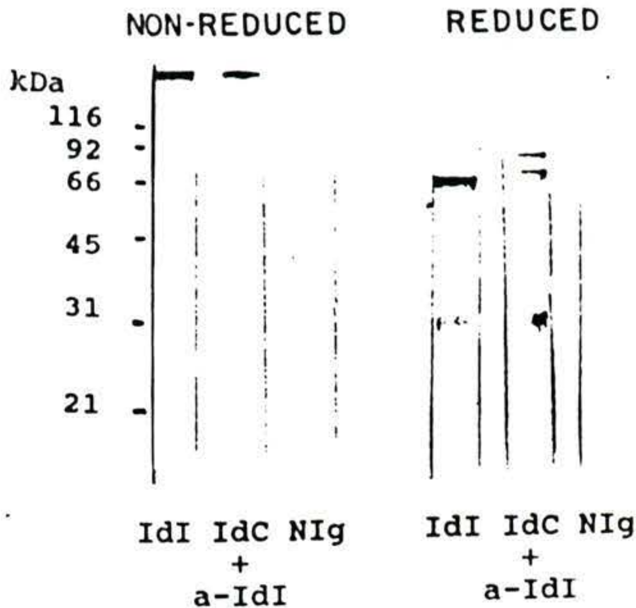

FIGURE 4. Western blot: samples of IdIp1 (*IdI*), IdCp1 (*IdC*), and NHIg (*NIg*) were electrophoresed in a 10% gel under reduced and nonreduced conditions, electroblotted to nitrocellulose and reacted with biotin-labeled anti-IdIp1 (*a-IdI*).

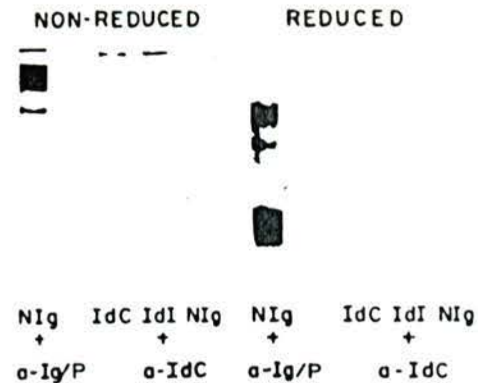

FIGURE 5. Western blot: samples of IdCp1 (*IdC*), IdIp1 (*IdI*), and NHIg (*NIg*) were electrophoresed in a 10% gel under reduced and nonreduced conditions, electroblotted to nitrocellulose, and reacted with biotin-labeled anti-IdCp1 (*a-IdC*). Two NHIg strips were directly incubated with anti-human Ig-peroxidase conjugate as reduced or nonreduced Ig control.

Table II
Anti-Id reactivities of reduced and nonreduced Id[a]

Id	Anti-IdIp2 Dilutions			Id	Anti-IdCp2 Dilutions		
	1/400	1/800	1/1600		1/400	1/800	1/1600
IdIp2	0.410	0.311	0.220	IdCp2	0.420	0.320	0.213
(r)IdIp2	0.320	0.280	0.140	(r)IdCp2	0.051	0.020	0.008

[a] ELISA plates were exposed to reduced ((r)IdIp2; (r)IdCp2), or nonreduced (IdIp2 and IdCp2) Id or NHuIg (3 μg/ml), washed, blocked, and incubated with increasing dilutions of biotin-labeled anti-Id. Anti-Id reactivity was detected by using extravidin-peroxidase conjugate. Data are reported as OD at 405 nm (Id reaction-NHuIg reaction).

preparation under reduced conditions (Fig. 5). This observation was further investigated in a more quantitative essay. Data generated using anti-IdIp2 in direct ELISA systems against both reduced ((r)IdIp2) and nonreduced (IdIp2) forms, confirmed that the Ids expressed on anti-EPI antibodies from IND patients remain largely recognizable after Ig reduction, i.e., many of the idiotopes recognized by the anti-IdI reagent are resistant to the effects of reduction (Table II). Furthermore, as seen in Figure 6, in (r)IdIp2/anti-IdIp2 competitive ELISA, IdIp2, and (r)IdIp2 are equally effectively in their competition of the reaction of anti-IdIp2 to the (r)IdIp2. It therefore appears that not only does reduction of IdI not destroy the majority of IND-related Id, it also does not create newly exposed Id that are recognized by these anti-Id reagents, which have led to more efficient inhibition. Upon calculation, the population of IND Id that is destroyed by reduction of Ig represents only 10 to 30% of the total IdI detected. In contrast, reduction of anti-EPI antibodies from CARD patients eliminates the ability of their predominant Ids to be recognized by anti-IdC (Table II), confirming the Western blot studies.

These findings of differential IdI vs IdC loss on reduction were also examined through the use of reciprocal competitive ELISA systems using IdIp2/anti-IdIp2 and IdCp2/anti-IdCp2 (Fig. 7). Upon reduction IdCp2 shows a strong decrease (85%) in its level of inhibitory ability in the CARD system. Thus, as in the direct recognition systems above, the IdC/anti-IdC competitive ELISA system shows that reduction of IdCp2 almost completely abolishes its inhibitory capacity, emphasizing the possible importance of conformational Id in the over all makeup of IdC. In parallel with the findings based on direct recognition (Table II), the competitive ELISA data indicate that about a third of the inhibitory activity of IdI in the IND system is lost upon reduction (Fig. 7). In the same experiment, the reduced Id preparations were also tested reciprocally as inhibitors in the noncomplementary Id/anti-Id systems. When tested in the IND system, (r)IdC was seen to have lost about 36% of its inhibitory activity, which paralleled the degree of loss suffered by (r)IdI (32%). As before IdI is an inefficient inhibitor in the CARD system and what competitor capacity it has is decreased about 40% upon reduction (Fig. 7). These results partially agree with the Western blot data, but they go further by providing the quantitative data. Assembly and analysis of these data demonstrate that IdIp2 can inhibit 100% in both the IdIp2/anti-IdIp2 and the (r)IdIp2/anti-IdIp2 systems, and also in the (r)IdCp2/anti-IdCp2 system (data not shown). Yet it only competes to a maximum of 43% in the normal (nonreduced) IdCp2/anti-IdCp2 system. It would seem there is a dominant population of Id in the CARD pool that is determined by the original tertiary conformation of the Ig molecules, which is probably distinct from, or poorly expressed by, the antibody molecules in the IND pool.

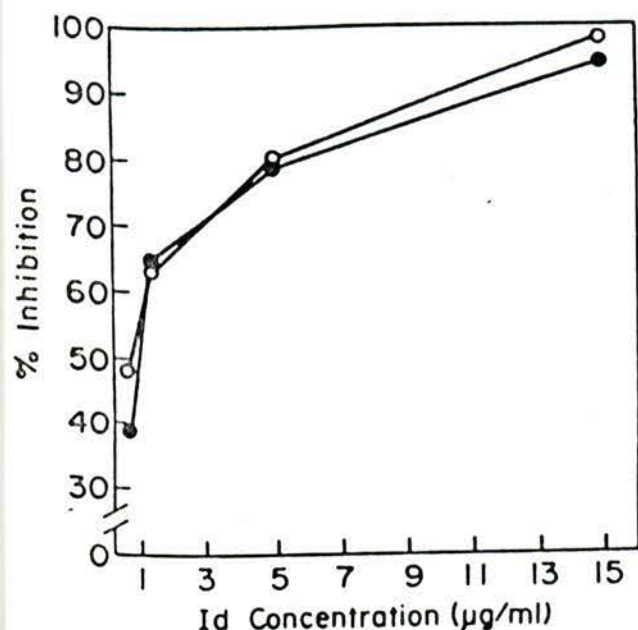

FIGURE 6. (r) IdIp2/anti-IdIp2 competitive ELISA. Wells of ELISA plates were coated with the reduced form of IdIp2 (*(r)IdIp2*). The inhibitory abilities of the reduced (○) and nonreduced (●) forms of IdIp2 were compared by preincubating them (0.32–15.00 µg/ml) with anti-IdIp2. The percent inhibition was calculated as described in the text. The experiment presented is representative of multiple repetitions and similar results are obtained with two different sets of Id/anti-Id combinations.

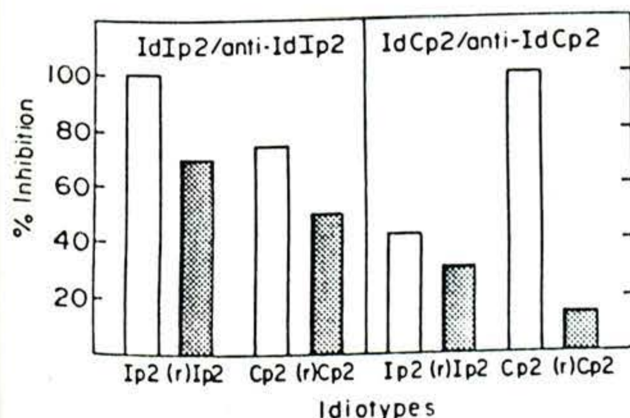

FIGURE 7. Comparative assays of the ability of reduced (*r*) and nonreduced Id preparations to inhibit binding in Id/anti-Id competitive ELISA assays. Biotin-labeled anti-IdIp2 and anti-IdCp2 were preincubated with reduced or nonreduced pooled Ids (20 µg/ml) and allowed to react with IdIp2- (*left panel*) or IdCp2- (*right panel*) coated ELISA plates. The percent inhibition was calculated as described in the text. The experiment presented is representative of multiple repetitions.

Discussion

We have previously reported that anti-EPI antibodies purified from the sera of IND patients differ from the anti-EPI antibodies purified from the sera of CARD patients. These differences have been both in the degree to which they stimulate anti-Id T cells (14, 15) and the mechanisms by which they stimulate this lymphocyte proliferation (16, 17). Several different IdC preparations have been generally more stimulatory for a given patients' PBMC than various IdI preparations, i.e., IdC usually stimulates the higher level of tritiated thymidine incorporation (14, 15). Also, stimulation of T cells from IND patients requires processing and MHC presentation, regardless of whether the source of the stimulating anti-EPI antibodies was an IND or CARD patient. In contrast, stimulation of T cells from CARD patients only requires processing and MHC presentation if the anti-EPI antibodies are from IND patients. IdC preparations are capable of stimulating T cells from CARD patients directly, without the need for either processing or presentation (16, 17).

Our data confirm that idiotypically different patterns are expressed by anti-EPI antibodies from these two different clinical sources, and the reduction data, in particular, strengthen our interpretation of the previous studies on Id processing and presentation. Many of the distinctive Id expressed on IdC-bearing antibodies are destroyed by reduction and alkylation, although very few of the Ids expressed on IdI antibodies are altered by this process. This situation would be most likely to occur if IdC Id are defined by conformational patterns of the antibodies involved.

Colley and Howard (18) and Colley (20) have speculated that this situation could provide an hypothetical basis for the pathogenesis of Chagas' disease. This hypothesis would still depend on autoimmunity for pathogenesis, but the target of the response would be a given set of Ids on a patients' own Ig and/or B cells rather than against cross-reactive epitopes shared by the organism and the host. This theory could fit with those of Stewart et al. (21) and Vaz and Faria (22) that suggest that inadequate immunologic connectivity could initiate pathologic autoimmune phenomena through pertubations of the dynamics of the self-referring Id-anti-Id network. Such hypotheses are currently pure speculation, and inadequate definitive data exist to resolve this at this time. Furthermore, if true, they would not be mutually exclusive of other mechanisms.

Our data showing correlations between clinical form and Id expression could also be explained by a hypothesis in which chronic infections in some patients would lead to a breakdown in self-tolerance and autoimmunity. In such a situation the new autoantibodies that would develop in CARD patients would express the novel IdC Id. Such IdC-expressing autoantibodies might be expected to have specificity for both given parasite Ag and certain host Ag. In one approach we have looked for such activity by a preliminary study using a range of concentrations of biotinylated IdIp, IdCp, and NHIg, tested by frozen-tissue immunohistochemistry on heart tissue from a patient who died of chronic Chagas' disease and an acute *T. cruzi*-infected mouse. Neither the IdIp nor the IdCp reacted with

the human or mouse cardiac tissues, but both antibody preparations were positive against the apparent *T. cruzi* organisms seen in the murine heart. This is not definitive proof against the possible presence of an autoantibody activity in IdC, but if such activities exists they appear not to be against self or altered-self epitopes that are exposed or preserved by snap-freezing and acetone fixation.

The somewhat overlapping nature of IdI and IdC observed in our study is most likely due to the sharing of many Ids among patients with different clinical forms, but a sharing that differs considerably in terms of proportionality. Although IdI contains many of those Id observed in IdC, it contains much less of them than does IdC. Individual IdC preparations varied considerably, in that 2 of 10 studied cases clearly containing many fewer IdC expressing antibodies than did the other eight. Whether this mimics or correlates with the spectrum of disease progression can not be determined based on the available clinical data.

Neither the origin nor the function of the Id differences defined by the rabbit anti-Id reagents described in this study are as yet clarified. Nevertheless, taken together with our earlier findings on differences in the abilities of IdI and IdC to stimulate patients' lymphocyte anti-Id responses, it seems clear that such differences exist, and their existance generally correlates well with the presence or absence of clinical Chagas' disease. This provides an initial means of evaluating differences between these individuals on a molecular level.

Acknowledgments

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TRABALHO #2

RUNNING HEAD:

$CD8^+$ CELLS DOMINATE HUMAN CHAGASIC CARDIAC LESIONS.

CHARACTERIZATION OF INFLAMMATORY INFILTRATES IN CHRONIC CHAGASIC MYOCARDIAL LESIONS: PRESENCE OF TNF-ALPHA$^+$ CELLS AND DOMINANCE OF GRANZYME A$^+$, CD8$^+$ LYMPHOCYTES.

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ABSTRACT

The inflammatory infiltrates in the heart lesions of chronic chagasic cardiomyopathy are composed predominantly of small lymphocytes with admixed macrophages, plasma cells and segmented leucocytes. The phenotypes of the lymphoid cells in these infiltrates of human Chagas' disease have not been previously detailed. We used a panel of monoclonals and polyclonal antibodies to immunohistochemically characterize the inflammatory cells in frozen and fixed cardiac tissues from autopsied patients with severe chronic chagasic cardiomyopathy. In all cases the inflammatory lesions were dominated by $CD8^+$ lymphocytes, many of which expressed granzyme A. A few macrophage-like cells which expressed tumor necrosis factor-alpha were observed in each case. Relatively few NK cells or B lymphocytes were found in the lesions. These findings in human chagasic lesions are compatible with concepts that involve cytolysis and fibrosis, and some new experimental findings that emphasize potential roles for $CD8^+$ cells in Chagas' disease.

Chagas' disease, caused by the intracellular protozoan parasite *Trypanosoma cruzi*, affects over 16-18 million people in South and Central America with rare sporadic cases in USA and Canada.\1-3/ Most of the morbidity and mortality in patients with chronic Chagas' disease is secondary to an inflammatory cardiomyopathy with attendant arrhythmias, conduction defects, congestive heart failure and/or thromboembolic events.\4,5/ These complications are thought to be directly related to death in about 15-20% of the infected individuals.\5/

The inflammatory lesions classically described in the hearts of individuals dying of chagasic cardiopathy are composed predominantly of small lymphocytes with admixed plasma cells, eosinophils, mast cells and macrophages, and are associated with myocellular destruction and fibrosis.\4, 6-8/ All areas of the heart, including the conduction pathways, can be involved.\5/ Although several autopsy series have described the light microscopic and ultra-structural features of human Chagas' disease,\6- 8/ further phenotypic and functional characterization of the cells in the heart lesions are needed to gain a better understanding of this pathological condition.

By using immunohistochemical techniques with a wide variety of phenotypic markers we have studied the lesions in six hearts of autopsied patients with chronic Chagas disease. The inflammatory infiltrates are composed predominantly of T cells, with few B cells and macrophages. The great majority of lymphocytes expressed the CD8 cell-surface marker. In conjunction with the findings of Higuchi\9/ these data provide the first indications of a major role for $CD8^{+}$ cells in human Chagas' disease. Most of the infiltrating cells in the heart lesions were shown to express granzyme A and a few in each lesion expressed tumor necrosis factor alpha (TNF-alpha). These two molecules are known to be involved in cytolytic, cell destructive events. These findings provide initial insights into potential

cytotoxic mechanisms involved in heart lesion formation in chronic Chagas' disease.

MATERIAL AND METHODS

Patients and samples:

Myocardial tissue samples were obtained from 6 patients with Chagas' disease autopsied at Faculdade de Medicina do Triângulo Mineiro, Uberaba, Minas Gerais, Brazil. Informed consent from family members was obtained prior to tissue procurement. The serologic diagnosis of *T. cruzi* infection was based on standard clinical laboratory complement fixation, hemagglutination and immunofluorescence tests, using serum and/or pericardial fluid. All of these patients had major, severe complications of chronic chagasic cardiomyopathy (Table 1). Five of them died from chagasic cardiomyopathy, while the death of the sixth (case #12), who suffered from severe congestive failure due to chronic chagasic cardiomyopathy, was more directly attributable to alcoholic liver disease. Tissue samples (1 cm x 1 cm x 4 cm) were collected from 3 different areas of each heart including the superior interventricular septum, the periapical left ventricule and the left ventricular free wall. Half of each specimen was processed in 10% neutral buffered formaldehyde solution and embedded in paraffin. Another portion was snap-frozen in liquid nitrogen and stored at -70\o/C. Both frozen and paraffin-embedded samples were sectioned at 5 µm, stained with hematoxylin-eosin and myocardial areas showing cellular infiltration on light microscopy were chosen for immunohistochemical staining.

Primary antibodies:

The monoclonal and polyclonal antibodies used for primary reagents in this study are listed in Table 2. Immunophenotyping on paraffin-embedded heart tissues was performed using mouse monoclonal antibodies for LCA/CD45, UCHL-1/CD45RO, L26/CD20, KP1/CD68 (Dako Corporation, Carpinteria, CA), Leu7/CD57 or rabbit polyclonal for

IgG, IgM, IgA, kappa and lambda chains (Becton- Dickenson, Mountainview, CA). Studies on frozen sections were done using mouse monoclonals including antibodies to CD2, CD3 (Dako); CD4, CD8 (Becton-Dickson); TNF-alpha (Boehringer Mannheim Corporation, Indianapolis, IN); and a rat monoclonal antibody to Granzyme A (T cell Diagnostics, Cambridge, MA). Mouse IgG (Sigma Chemical Company, St. Louis, MO) and rat IgM (Accurate Chemical & Scientific Corporation, Westbury, NY) were used as negative primary antibody controls.

Immunohistochemistry on paraffin-embedded heart tissues:

Paraffin-embedded heart tissues from all 6 autopsied patients were used for this study. Five micron sections were deparaffinized through xylene and graded alcohols and then incubated in 1% hydrogen peroxide 30% in absolute methanol for 30 min to inhibit endogenous peroxidase activity. After rehydration in Tris buffer saline solution pH 7.2 (TBS), the slides were incubated with 2% normal swine serum (NSS) (Sigma) in TBS for 15 min and, subsequently with primary antibodies (Table 2) for 1 hr, followed by a second step incubation with peroxidase-conjugated rabbit anti- mouse antibodies (DAKO) for 45 min. Between incubations the slides were washed in TBS for 15 min. For IgG, IgM, IgA, kappa and lambda, the immunoperoxidase PAP technique was used. After rehydration in phosphate buffer saline solution pH 7.2 (PBS), the slides were incubated with 10% NSS in PBS for 10 min. The primary antisera were diluted in 1% NSS in 0.4% Triton X-100 in PBS and loaded on the slides overnight. The secondary antibody swine anti-rabbit (Dako) in 10% NSS was incubated for 30 min, followed by the conjugated rabbit peroxidase- anti-peroxidase (DAKO) in 1% NSS for 30 min. In each case peroxidase activity was demonstrated by incubation with 3-3'-diaminobenzidine (Sigma) and hydrogen peroxide for 10 min. Slides were counterstained with Gill's hematoxylin (Sigma), dehydrated in graded alcohols and mounted in synthetic mounting media.

Immunohistochemistry on frozen sections:

Frozen heart tissues from 5 autopsied patients were used in these studies. Five micron sections were fixed in acetone for 10 min, incubated with primary antibody appropriately diluted in PBS-BSA 2%, and then sequentially with peroxidase-conjugated rabbit anti-mouse IgG (Dako) 1:40 or peroxidase-rabbit anti-rat immunoglobulin (Dako) 1:40. Incubations were of 1 hr each and between incubations the slides were washed with PBS for 3 min. Additionally, frozen lymph node tissues from two of the autopsied patients were also processed as described above.

Expression of TNF-alpha was investigated in these 5 frozen heart tissues using an extravidin/biotin kit (Sigma). Slides were incubated overnight with anti-TNF-alpha antibody (Boehringer-Mannheim) diluted in PBS-BSA 2%. The reactions were developed with 3-3' -diaminobenzidine (Sigma) and hydrogen peroxide for 10 min, and enhanced with cooper sulfate 0.5% for 5 min. Nuclear counterstaining was obtained with Mayer's Hematoxylin (Sigma).

Assessment of results:

Appropriate negative and positive control slides were run for each case. These controls were available from non-chagasic cases previously characterized by immunohistochemistry or flow cytometry in the Immunopathology Service, Vanderbilt University Hospital. Enumeration of cells was done by light microscopy under oil (1000 X original magnification). A minimum of 100 mononuclear cells (lymphocytes and macrophages/monocytes) on a single slide were counted per patient. For anti-TNF-alpha and anti-$CD68^+$ cells stained sections at least 300 cells per slide were counted. The fields selected for study contained inflammatory infiltrates, each with a minimum of 25 cells.

RESULTS

Fibrosis associated with a heavy inflammatory infiltrate was observed in all 6 cases studied. Table 3 shows the results of the characterization of inflammatory cells in paraffin-embedded heart tissues for the following phenotypes: pan-leucocyte (LCA/CD45), T lymphocyte (UCHL1/CD45RO), B lymphocyte (L26/CD20, IgG, IgM, IgA, kappa and lambda chains), NK cells (CD57) and macrophage (KP1/CD68). In each case there was a clear predominance of CD45RO$^+$ T lymphocytes (67 - 80%), with few B cells (2- 8%) and macrophages (<1-8%). A few cells resembling plasma cells, that stained with antibodies to IgG, IgM, IgA, anti-kappa or lambda chains were observed in every case, and were prominent in case #21. Antibody against the NK marker, CD57/Leu7, stained from <1 to 7% of the total cells in the inflammatory infiltrates (Table 3).

Frozen section studies showed that most of the cells in the inflammatory infiltrates of heart lesions expressed CD2 and CD3 Pan T cells markers. Figures 1 and 2 show that the great majority of T cells bore the CD8 marker while only a small percentage of T cells stained with anti-CD4. The dominance of CD8$^+$ T cells was observed in every case (Table 4). The mean ± SD of the CD4:CD8 ratio within these lesions was 0.36 ± 0.03. Concomitant studies of lymph nodes from two of these patients showed a normal CD4 and CD8 distribution with predominance of CD4$^+$ lymphocytes (Aut. #6, CD4/CD8 = 1.7; Aut. #12, CD4/CD8 = 2.0).

When frozen sections were stained with a monoclonal antibody reactive with granzyme A (Figure 3), a large number of positive cells (38-68%) were noted in the inflammatory infiltrates. It seems likely that most granzyme A$^+$ cells are CD8$^+$ lymphocytes. Although there is no direct evidence for this allegation, in every case the percentage of granzyme A$^+$ cells was always higher (1.6-2.7 fold) than the percentage of CD4$^+$ lymphocytes. The percentage of granzyme A$^+$ cells closely paralleled the percentage of CD8$^+$ cells in most cases.

Cells expressing TNF-alpha, most of which had the morphology of macrophages, were present in every case (Figure 4). The percentage of TNF-alpha$^+$ cells ranged from 2.6 to 8.1%.

TABLE 1

Study group

Case No	Major clinical pathologic diagnoses
6	CCC*/ with congestive heart failure
7	CCC*/ with sudden death
10	CCC*/ with congestive heart failure
12	EtOH cirrhosis, CCC*/ with congestive heart failure and pneumonia
15	CCC*/ with congestive heart failure and megacolon
21	CCC*/ with cerebrovascular accident and acute pyelonephritis

*/CCC: chronic chagasic cardiomyopathy

TABLE 2

Primary antibodies utilized in this study

Antibody specificity	Main reactive population	Working dilution
CD45 (LCA)	majority of all leucocytes	1:10
CD45RO (UCHL1)	T cells (activated/memory)	1:40
CD20 (L26)	B cells	1:100
IgG	B cells	1:2000
IgM	B cells	1:2000
IgA	B cells	1:2000
k light chain	B cells	1:2000
light chain	B cells	1.2000
CD68 (KP1)	macrophages/monocytes	1:40
CD3	T cells	1:40
CD2	T cells, NK cells	1:30
CD4	T helper/inducer, macrophages/monocytes	pre-diluted
CD8	supressor / cytotoxic T lymphocyte	pre-diluted
CD57 (Leu7)	NK cells	pe-diluted
Granzyme A	cytotoxic T cells	1:20
TNF-alpha	activated macrophages	1:40

TABLE 3

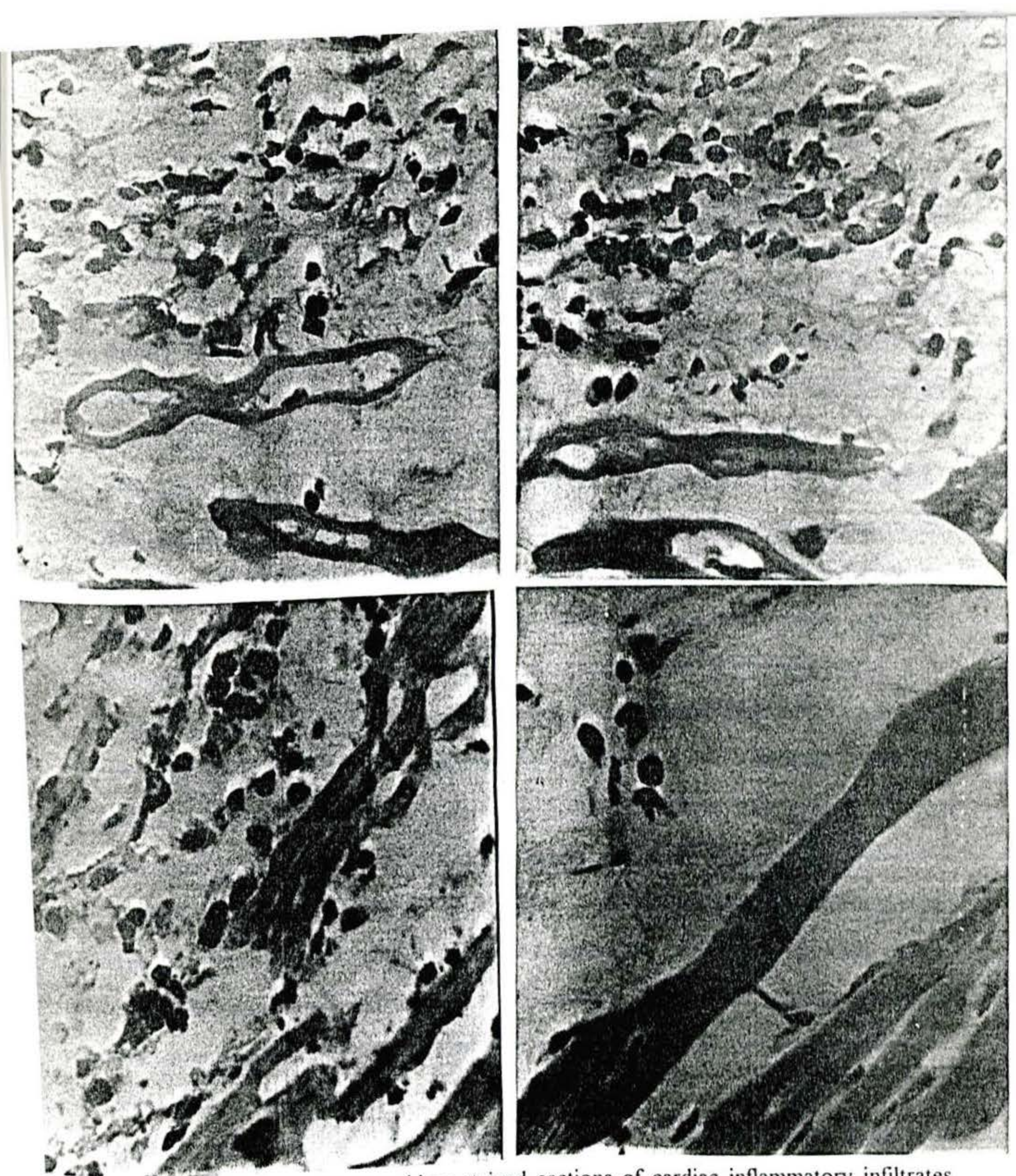
Characterization of inflammatory cells in formalin-fixed paraffin-embedded heart tissues

	Percentage of positive cells									
Case No	CD45 (LCA)	CD45RO (UCHL1)	CD57 (Leu7)	CD68 (KP1)	CD20 (L26)	kappa chain	lambda chain	IgG	IgM	IgA
6	89%	80%	2%	<1%	4%	<1%	<1%	<1%	<1%	<1
7	87%	75%	1%	7%	6%	4%	2%	4%	3%	1
10	92%	77%	<1%	2%	2%	3%	3%	3%	1%	<1
12	92%	67%	5%	3%	8%	5%	2%	6%	<1%	3
15	85%	67%	7%	8%	3%	4%	2%	2%	<1%	3
21	94%	72%	3%	6%	4%	7%	4%	6%	1%	2

TABLE 4

Characterization of inflammatory cells in frozen heart tissues

	Percentage of positive cells					
Case No	CD3	CD2	CD4	CD8	Granzyme A	TNF-alpha
6	76%	65%	24%	70%	39%	2.6%
10	87%	87%	25%	69%	52%	5.0%
12	84%	88%	25%	68%	68%	2.6%
15	64%	58%	21%	64%	38%	8.1%
21	70%	67%	28%	68%	65%	5.1%



FIGURES 1-4: Immunoperoxidase stained sections of cardiac inflammatory infiltrates with antibodies to CD4 *(Figure 1 - upper left)*, CD8 *(Figure 2 - upper right)*, granzyme A *(Figure 3 - lower left)* and TNF-alpha *(Figure 4 - lower right)*. (Mayer's hematoxylin counterstained, x 600).

DISCUSSION

Our study shows that the dominant cell type in the inflammatory lesions of human chronic chagasic cardiomyopathy is a T lymphocyte expressing the CD8 cell surface-marker. In a similar, concurrent study Higuchi\9/ has found very similar results. We have observed that the vast majority of the $CD8^+$ T cells in our study appear to be CD3+ and do not express CD57/Leu7, suggesting that they are T cells and not NK cells.

In addition we found that a sizable number (38-68%) of these cells in the chronic chagasic cardiomyopathy infiltrates express granzyme A. Previous studies by others have demonstrated the usefulness of this molecule as a marker for cytolytic lymphocyte responses *in vivo*.\10-13/ Granzyme A is a serine esterase found in the granules of activated killer cells and absent in resting T lymphocytes.\14/ It has been proposed that released granzyme A may induce endonucleolytic degradation of target cell DNA and may have proteolytic activity on endothelial-derived extracellular matrix proteins contributing to the migration of lymphocytes into tissues.\15/ Therefore, the demonstration of granzyme A^+ lymphocytes in the heart lesions of chronic chagasic cardiomyopathy provides strong evidence for the involvement of cytotoxic events in myocardial tissue damage. This concept agrees with previous experimental studies |in vitro| which demonstrated the cytotoxic interaction of *T. cruzi*-sensitized lymphocytes with parasitized and non-parasitized heart cells from mice and rabbits\16,17/ and also with human heart cells.\18/

$CD8^+$ T cells have classically been associated with viral infections, but in some cases intracellular bacteria\19,20/ or protozoa\21-23/ have also been shown to induce specific $CD8^+$ T cells. It is well to remember that *T. cruzi* actually replicates intracellularly in the cytoplasm of infected cells, and thus might be expected to be able to have some of its antigenic epitopes expressed in relationship to Class I MHC molecules.\24,2/ Recent |in situ| studies indicated that $CD8^+$ cells predominate in the acute chagasic heart lesions in

rats\26/ and mice\27/. Still other findings in experimental models suggest a major role for $CD4^+$ T cells in pathogenesis during the chronic *T. cruzi* infection.\28-30/ Thus, the relative contribution of $CD4^+$ and $CD8^+$ T cells in the development of experimental chagasic cardiomyopathy is still open to question.

Our data do not address the question of whether the $CD8^+$ cells seen in severe human chronic chagasic cardiomyopathy are mounting a specific anti-*T. cruzi* response, or an autoimmune response, or whether their activation has occurred locally or in a remote lymphoid organ followed by recruitment to this site. The presence of the intact parasite in chronic lesions is still argued.\6,7,31/ In this regard we have recently provided evidence by PCR amplification that a *T. cruzi*-specific repetitive DNA sequence is consistently found in all inflammatory lesions examined from patients with chronic chagasic cardiomyopathy.\32/

Another significant observation in our study is that cells with the morphology of macrophages in human chronic chagasic cardiomyopathy lesions express TNF-alpha. TNF-alpha is not only a marker of cytolytic events, but has wide ranging effects, including cell protection and the promotion of cell differentiation.\33,34/ The association of TNF-alpha with the pathology of some parasitic disease.\35-38/ and its direct involvement in the process of fibrosis and thromboembolic events,\34/ makes the demonstration of TNF-alpha+ cells in heart lesions of chronic chagasic cardiomyopathy, worthy of note in the study of this chronic pathology. TNF-alpha has been also reported to be important in the up-regulation of the expression of adhesion molecules such as ICAM-1.\34,39/ If this occurs in chagasic heart tissues, it would provide a mechanism for the localization of inflammatory cells, which could then contribute to the amplification and maintenance of these lesions. Further study of the roles of the adhesion molecules in the pathogenesis of these lesions is indicated.

Our studies show that $CD8^+$ lymphocytes expressing granzyme A and cells expressing TNF-alpha are present in the lesions of chronic human Chagas' disease. These findings

strongly support a role for cytotoxic immune mechanisms in the development of the heart lesion in this chronic pathology. These data address the effector functions responsible for lesion formation, but do not address the target of such cytotoxicity. It remains fully possible that these cytotoxic activities are being mounted either against the parasite and/or in an autoimmune manner. Further studies on TCR repertoire usage and definition of the T cell specificities involved will be necessary to address these questions directly.

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TRABALHO #3

RUNNING HEAD:

MHC AND ADHESION MOLECULES IN CHAGASIC HEARTS

EXPRESSION OF MHC ANTIGENS AND ADHESION MOLECULES IN HEARTS OF PATIENTS WITH CHRONIC CHAGAS' DISEASE

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ABSTRACT

We have previously reported that heart lesions in patients with chronic cardiac Chagas' disease are composed predominantly of granzyme A+, cytolytic CD8+ T lymphocytes. We now pursue this study in the immunopathology of chronic chagasic cardiomyopathy by investigation of the expression of HLA antigens, and adhesion molecules in the hearts of 7 chagasic patients with cardiac disease, 2 asymptomatic chagasic patients and 7 normal controls. Comparative immunohistochemical analyses show that HLA ABC antigen expression is up-regulated on the myocardial cells of chagasic patients with chronic cardiomyopathy, suggesting a possible role for these cells as targets for the CD8+ cytolytic lymphocytes dominant in these lesions. HLA DR antigens are not observed on myocardial cells, but is consistently up-regulated on the endothelial cells in the hearts of patients with chronic chagasic cardiomyopathy. ICAM is expressed by endothelial cells of both chagasic and non-chagasic individuals, but ELAM was detected only on vessels of hearts from chagasic patients who had chronic cardiomyopathy. Most of the lymphocytes in these lesions express LFA-1, CD44 and VLA-4, and a few display weak expression of LFA-3. We propose that the expression of these adhesion molecules and MHC antigens by endothelial cells, myocardial cells and lymphoid cells in these lesions contribute to the pathogenesis of chronic chagasic cardiomyopathy.

Chagas' disease is caused by *Trypanosoma cruzi* an intracellular hemoflagelate protozoa.\1/ Following acute infection most patients enter the so called indeterminate phase, which oftens presents only only serologic evidence of infection. After a long asymptomatic period of up to 20 years or more, 15-20% of patients develop clinical cardiac disease that is usually fatal.\2,3/ The histopathology of chronic chagasic myocarditis shows a predominantly mononuclear cell infiltrate closely associated with damaged cardiac fibers.\4,5/ The composition of the inflammatory infiltrate has recently been characterized immunohistochemically as containing predominantly granzyme A+, CD8+ T cells, some CD4+ cells, admixed with a few macrophages which express TNF-alphaa.\6/ Granzyme A is a serine esterase known to be involved in cytolytic mechanisms.\7/ These findings suggest a mechanism for myocardial destruction involving cell mediated cytotoxicity. Given the essential role of HLA molecules in antigen presentation\8/ and the pivotal importance of cell adhesion molecules in the regulation of lymphocyte trafficking,\9/ we decided to immunohistochemically examine the expression of HLA antigens and selected adhesion molecules and their ligands in the hearts of patients with chronic chagasic cardiomyopathy.

MATERIALS AND METHODS

Patient population and tissue collection:

Specimens of heart tissues were obtained by autopsy at Faculdade de Medicina do Triângulo Mineiro, Uberaba, Brazil, with the consent of family members. The diagnosis of Chagas' disease was based on both clinical history and confirmatory positive serologic testing by complement fixation, hemagglutination and immunofluorescence tests on serum and/or pericardial fluid. In this study we used tissue from 9 seropositive cases, all of whom have been reported previously.\6,10/ Seven patients [(cases #4 (51 years old, female), #6 (38 years old, male), #7 (24 years old, male), #10 (47 years old, male), #12 (40 years old,

male), #15 (45 years old, male) and #21 (38 years old, male)] had severe complications of chronic chagasic cardiomyopathy with congestive heart failure in 4 (cases #6, #10, #12 and #15), arrhythmias in 2 (cases #4 and #7) and an embolic cerebrovascular accident in 1 (case #21). Six of these 7 died from chagasic cardiomyopathy, while the death of the seventh (case #12), who did suffer from severe chronic cardiomyopathy, was primarily attributed to alcoholic liver disease. Two of the 9 seropositive cases [cases #18 (63 years old, female), and #20 (79 years old, female)] did not have clinical findings attributable to chronic chagasic cardiomyopathy and were thus classified as indeterminate patients. One of them (case#18) had heart disease with a prosthetic aortic valve. The control group consisted of 7 seronegative non-chagasic individuals [cases #1 (25 years old, male), #5 (44 years old, female), #8 (33 years old, male), #9 (30 years old, male), #16 (60 years old, male), #19 (50 years old, female), #22 (49 years old, female). These individuals did not present any clinical or histopathologic features of Chagas' disease.

Heart tissues from the cardiac apex, left ventricular free wall, and interventricular septum were rapidly frozen in liquid nitrogen and stored at -70\o/C. All autopsy specimens were sectioned and stained with hematoxylin-eosin staining.

Immunohistochemistry:

Investigation of expression of monomorphic portions of human MHC class I (HLA ABC) antigens and adhesion molecules were carried out using an indirect immunoperoxidase procedure. Briefly, 5 μm cryostat serial sections were cut, dried, acetone fixed, and incubated with one of several primary mouse monoclonal antibodies (Table 1) for 90 minutes. The second step consisted of incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako Corporation, Carpinteria, CA) for one hour. The enzyme activity was developed using 3-3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO). Sections were counterstained with Mayer's hematoxylin (Sigma). Negative controls were performed by substituting normal mouse immunoglobulin (Sigma) for the primary

antibody. MHC class II (HLA DR) expression in the heart tissues was investigated using an avidin-biotin peroxidase system. Frozen sections were treated with biotin-labeled mouse anti-monomorphic human HLA DR monoclonal (Dako) and incubated with avidin-peroxidase complex (Sigma). The reactions were developed with diaminobenzidine and the slides were counterstained with Mayer's hematoxylin (Sigma).

The reactivity of the various monoclonals was graded by the intensity of reaction visible. A value of 3 denotes intense reaction easily seen at 40x, while a score of 2 indicated that intense staining was only observed at 100x and a value of 1 means positivity appreciated only at 400x. Each specimen was scored individually by two observers unaware of the origin of the tissue, and the arithmetic mean value was calculated. Positive control slides were run for each case. These controls consisted of tissues from non-chagasic cases in which the expression of HLA antigens and adhesion molecules have been previosly well characterized.

RESULTS

Expression of MHC antigens:

Sixteen autopsy heart tissues (9 from autopsied patients with positive serology for Chagas' disease and 7 from non-chagasic controls) were examined for the MHC antigens expression. Moderate HLA ABC staining was observed on endothelial cells in tissues from both, chagasic and non-chagasic individuals (Figures 1 and 2). Myocardial cells of non-chagasic patients did not express detectable HLA ABC antigens on their surface (Figure 1), except one case (case #9), in which weak expression (grading score = 0.5) of this antigen was observed. In the 2 patients with positive serology for Chagas' disease but no symptomatology of chagasic cardiomyopathy (Indeterminate cases: #18, and #20) class I antigen expression on myocytes was likewise not observed (Table 2). In contrast, 6 of 7 patients with chronic chagasic cardiomyopathy expressed HLA ABC on their myocardial cells, with grading scores ranging from 1.5 to 3.0 (Figure 2, Table 2). This occured

primarily in areas which contained inflammatory foci.

The staining for Class II antigens (HLA DR) expression in the cardiac tissue of chagasic and non-chagasic patients is summarized in Table 3. HLA DR was always detected on endothelial cells of small and large vessels of both chagasic and non-chagasic tissues. However, in specimens from individuals with chronic chagasic cardyomiopathy HLA DR was strongly expressed on both small and large vessels, while in most non-chagasic patients it was only faintly observed (Figures 3 and 4). The grading scores for small vessels were 0.5-1.0 in control cases, and 2.0-3.0 in patients with chronic chagasic cardiomyopathy. For large vessels the grading scores were 0.5-1.5 (except for case #5) in control cases and 2.5-3.0 in patients with Chagas' disease. Two indeterminate cases were examined and one of them (case #18) also had aortic valvular disease attributable to causes other than Chagas' disease. Both indeterminate cases presented up-regulation of HLA DR expression on large vessels but only one (case #18) showed strong staining on small vessels (Table 3). HLA DR expression was not detected on myocytes of any of the cases examined (Figures 3 and 4).

Expression of adhesion molecules:

Antibodies against two adhesion molecules, ELAM (endothelial leukocyte adhesion molecule) and ICAM-1 (intercellular adhesion molecule), stained endothelial cells. ICAM-1 was detected to varying degrees on all tissues examined, without any obvious difference between chagasic and non-chagasic cases (Table 4). In samples from chagasic individuals, strong expression of ICAM-1 was also evident on lymphocytes in inflamatory infiltrates (data not shown). Weak expression of ELAM was observed on the endothelial cells of both small and large vessels of chagasic patients with cardiac disease, preferentially in areas with inflammation, but it was absent from tissues of most control and the two indeterminate cases (Table 4, Figures 5 and 6).

The vast majority of lymphocytes in the chronic chagasic heart lesions stongly expressed both CD11a and CD18 chains of Lymphocyte Function Antigen-1 (LFA-1) (Figure 7). Most of the lymphocytes in these inflammatory infiltrates strongly expressed also CD44 (lymphocyte homing receptor) (Figure 8) and moderately expressed Very Late Antigen-4 (VLA-4) (CD29 / CD49d) (Figure 9). In contrast, only a few cells in these lesions expressed LFA-3, and they did so weakly (not shown). Expression of CD44 was also observed on endothelial cells of chagasic (Figure 8) and non- chagasic individuals (not shown).

TABLE 1

Mouse anti-human monoclonal antibodies

Specificity	Commercial Origin	Dilution
HLA ABC	Dakopatts	1:50
HLA DR - biotin	Becton-Dickinson	1:100
ICAM-1 (CD54)	Becton-Dickinson	1:50
ELAM	Becton-Dickinson	1:25
CD44	Janssen Biochimica	1:50
LFA1 (CD18)	Janssen Biochimica	1:25
LFA1-alfa (CD11a)	Dakopatts	1:20
LFA3 (CD58)	Becton-Dickinson	1:50
VLA-4	Upstate Biotechnology	1:20

TABLE 2

Histologic grading of inflammation\#/ and HLA ABC/ expression by myocardial cells on heart tissues of patients with Chagas' disease*

Clinical Form	Case No	Inflammation	HLA ABC expression on myocardial cells
Indeterminate	18	0.5	0.0
	20	0.0	0.0
Cardiac	4	0.5	0.0
	6	2.5	3.0
	7	2.0	1.5
	10	2.0	2.0
	12	3.0	3.0
	15	3.0	3.0
	21	1.5	2.0

\#/Focus of inflammation was defined as previouslly described\6/

*/Histologic grading of HLA ABC expression was performed as described in the Matherial and Methods.

TABLE 3

HLA-DR expression on heart tissues

		Histologic grading score*/	
Clinical Form	Case No	Small vessels	Large vessels
Non-chagasic	01	1.0	0.5
	05	0.5	3.0
	08	0.5	1.5
	09	0.5	0.5
	16	1.0	1.5
	19	0.5	0.5
	22	1.0	0.5
Indeterminate	18	2.0	2.5
	20	1.0	2.5
Cardiac	04	2.0	2.5
	06	3.0	3.0
	07	2.5	2.5
	10	2.5	2.5
	12	2.5	3.0
	15	2.5	3.0
	21	2.5	3.0

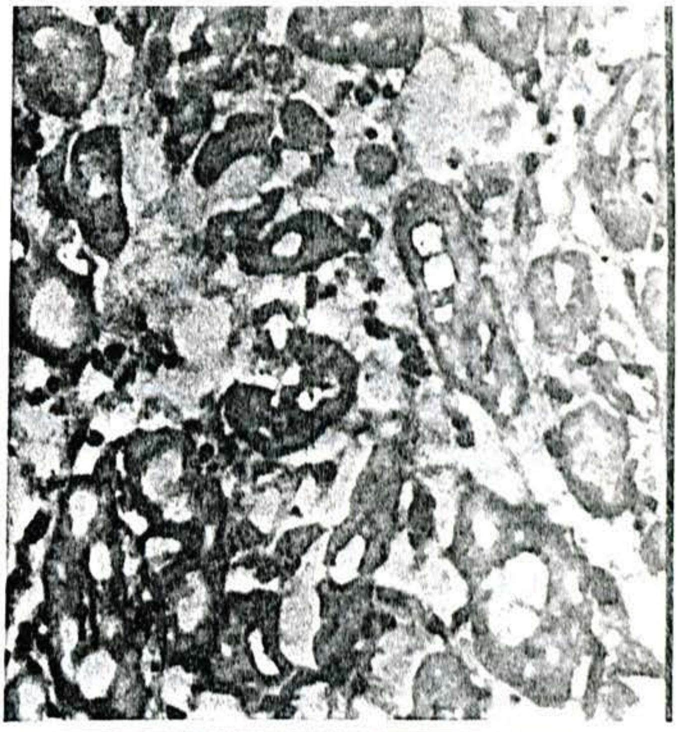
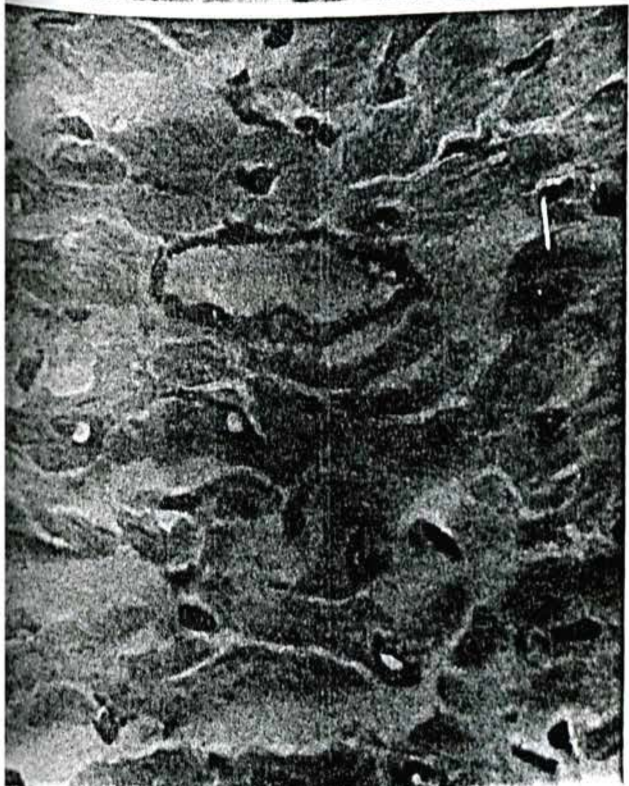
*/Histologic grading was performed as des- cribed in the Materials and Methods.

TABLE 4

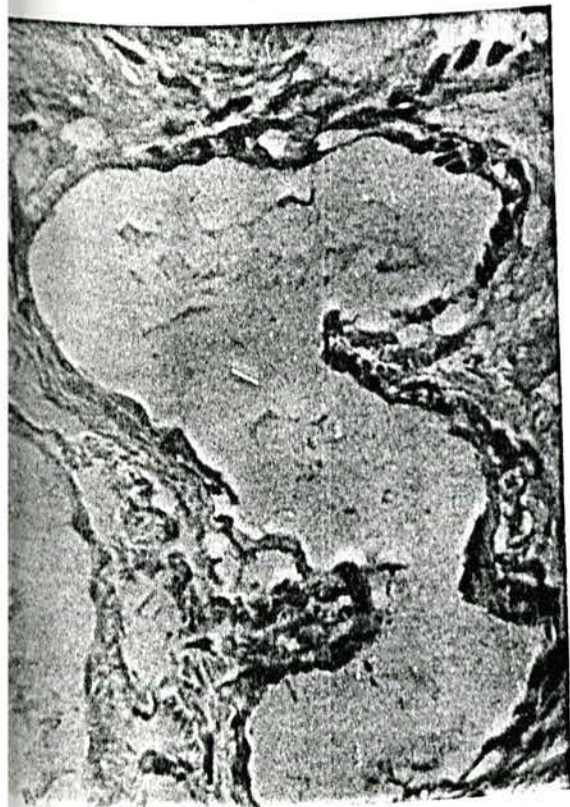
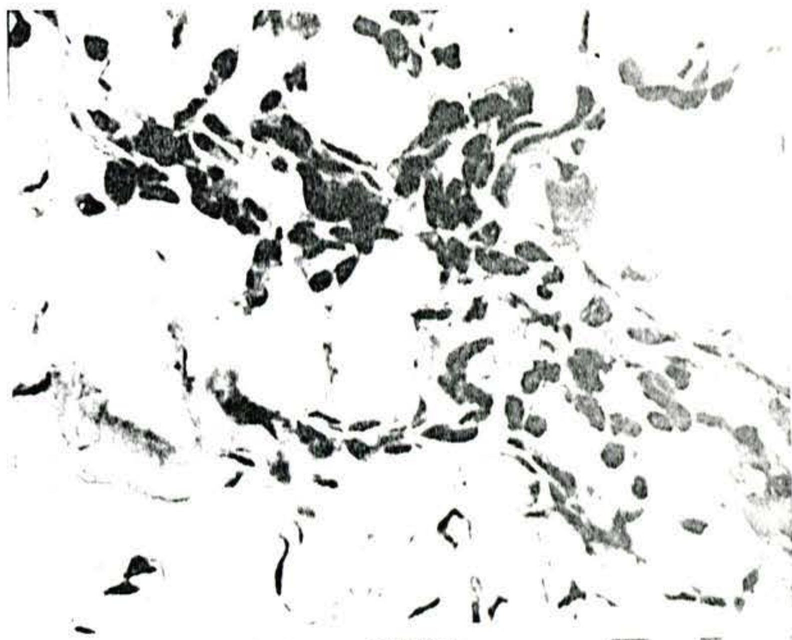
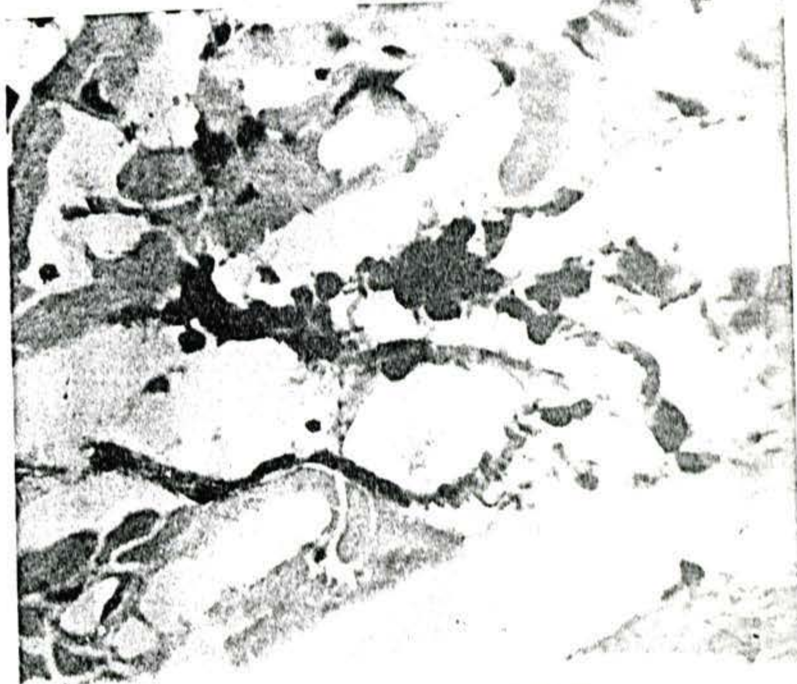
ICAM and ELAM expression on heart tissues

Clinical Form	Case No	Histologic grading score*/			
		Small vessels		Large vessels	
		ICAM	ELAM	ICAM	ELAM
Non-chagasic	01	2.0	0.5	3.0	0.5
	05	3.0	0.0	2.0	0.0
	08	2.0	0.0	1.5	0.0
	09	1.0	0.5	2.5	0.5
	16	1.5	0.0	1.0	0.0
	19	2.0	0.0	1.0	0.0
	22	2.0	0.0	2.0	0.0
Indeterminate	18	1.0	0.0	1.0	0.0
	20	2.0	0.0	1.5	0.0
Cardiac	04	3.0	0.5	3.0	0.5
	06	2.5	0.5	2.0	0.5
	07	2.0	1.0	2.5	1.0
	10	2.0	1.0	2.0	1.0
	12	2.0	1.5	2.0	1.5
	15	2.5	1.5	2.5	1.5
	21	3.0	1.0	3.0	1.0

*/Histologic grading was performed as described in the Materials and Methods.

FIGURES 1-4: Immunoperoxidase stained sections of human hearts stained with antibodies to MHC antigens. |Figure 1| (upper left, non-chagasic individual) and |Figure 2| (upper right, cardiac chagasic patient) were reacted with an antibody to HLA ABC. (x400). |Figure 3| (lower left, non-chagasic individual) and |Figure 4| (lower right, chagasic patient) were reacted with an antibody to HLA DR. (x600) (Mayer's hematoxylin counterstained).

FIGURES 5-9|: Immunoperoxidase stained sections of human hearts stained with antibodies to ELAM |(Figure 5 - upper left, chagasic patient - and Figure 6 - lower left, non-chagasic individual)| (x600). Immunoperoxidase stained sections of human heart tissues from cardiac chagasic patients stained with antibodies to LFA-1 |(Figure 7, upper right)|; CD44 |(Figure 8, middle right)|; and VLA-4 |(Figure 9, lower right)|. (x600, Mayer's hematoxylin).

DISCUSSION

In agreement with other studies\11/,12/ we have demonstrated that normal human cardiac myocardial cells express low or no detectable levels of Class I MHC (HLA ABC) antigens and do not express Class II MHC (HLA DR) antigens. This is also true for patients who are serologically positive for infection with *T. cruzi* but are asymptomatic. In contrast, myocardial cells in 6 of 7 patients with chronic chagasic cardiomyoopathy reacted strongly with antibody against HLA ABC. Thus, this study provides the first evidence that chronic chagasic cardiomyopathy is associated with up-regulation of class I MHC antigen expression by myocardial cells. In light of our previous studies showing the dominance of CD8+, granzyme A+ lymphocytes in these chagasic heart lesions,\6/ the current study would support the hypothesis that cardiac myocytes expressing high levels of Class I antigens, and perhaps exogenous or endogenous antigen peptides, could be targets for cytotoxic T lymphocytes. This could contribute to the myocardial damage observed in chronic chagasic cardiomyopathy.

Increased expression of MHC class I antigens by myocardial cells has been demonstrated in human tissues undergoing allograft rejection,\11-15/ certain viral diseases\16,17/ and other inflammatory states.\18/ It is possible that *Trypanosoma cruzi* infecting the cardiac myocytes would induce MHC class I up-regulation in these cells, similar to that seem in some viral infections. We have previouly presented evidence for *T. cruzi* DNA sequences within these inflammatory lesions.\10/ Considering the fact that the level of HLA ABC expression on myocardial cells was correlated with the presence of inflammatory infiltrates, it could also be possible that the activated lymphocytes and monocytes might play an important role in the induction of class I antigen expression by these cells. This would be in agreement with the demonstration that Class I antigen can be up-regulated |in vitro| in human cardiac myocytes by cytokines such as IFN- gamma, IL-1, IL-4 and TNF-alpha \19/ We have previously identified cells expressing TNF-alpha in heart tissues from patients with

chronic chagasic cardiomyopathy,\6/ but our studies can not resolve the question of cause-and-effect relationships in this setting.

We did not detect HLA DR expression on myocardial cells of either chagasic or non-chagasic patients, but we did observe its presence on the endothelial cells of all tissues examined. It was most strongly and consistently expressed on small and large vessels of tissues from patients with chronic chagasic cardiomyopathy. Only a few HLA DR+ macrophages and scarce HLA DR+ lymphoid cells were observed in the chagasic heart lesions. It has been shown that IFN-gamma treated human endothelial cells acquire the capacity to present antigens to T cells in culture, leading to effective T cell activation.\20,21/ Other studies also suggest that endothelial cells can present alloantigens to circulating T cells in the transplantation setting, thereby initiating rejection reactions.\22/ Although these proposed antigen-presenting functions of endothelial cells have not yet been established |in vivo|,\22/ our data showing the up-regulation of HLA DR on endothelium and the scarcity of HLA DR+ lymphocytes or macrophages, raise the possibility that endothelial cells in the hearts of chagasic patients could be the primary antigen-presenting cell to those CD4+ helper T cells that arrive at these sites of inflammation.

Endothelial cells can also participate in inflammatory processes by recruiting lymphocytes into inflammatory sites independent of specific antigen. This process is thought to involve recognition by lymphocytes of adhesion molecules on vessel endothelial cells at sites of inflammation.\22/ We examined the endothelial cells in cardiac tissues of chagasic and non-chagasic patients for the expression of several adhesion molecules including ICAM-1 and ELAM. ICAM-1 expression is widely distributed and may be present or induced on almost all cell types.\23/ ELAM is expressed primarily by IL-1 or TNF-treated endothelial cells, but not by resting endothelial cells.\24,25/ Other authors had previously reported the expression of ICAM-1 by endothelial cells in hearts of normal individuals.\26/ Our results confirmed this observation and in addition showed that ICAM-1 is also consistently expressed by endothelial cells in the hearts of chagasic patients. In contrast, ELAM was not

detected on the heart tissues of non-chagasic or asymptomatic chagasic patients, but was consistently, albeit weakly, expressed by the small vessel endothelial cells in patients with chronic chagasic cardiomyopathy. As already mentioned, in these same tissues we had previously demontrated the presence of some cells producing TNF-alpha,\6/ and this might explain the presence of ELAM+ endothelial cells.

Adhesion molecule ligands were studied on lymphocytes. LFA-1, VLA-4, and CD44 mediate the adhesion of lymphocytes to endothelium activated by inflammatory cytokines\22,27/ and are therefore considered to be important in the localization and infiltration of cells into sites of inflammation. We observed that most of the infiltrating cells in inflammatory lesions expressed LFA-1, VLA-4, and CD44 and a few weakly express LFA-3. LFA-1 is the ligand for ICAM-1 and VLA-4 is the ligand for fibronectin or VCAM (vascular adhesion molecule).\28/ CD44 has been shown to be a ligand for the extracellular matrix constituent hyaluronic acid\29,30/ and may, in addition, have binding specificity for collagen and fibronectin.\9,31/ CD44 may therefore be important also for adhesion to, or migration through, the extracellular matrix in these tissues.\30/ Thus, it is likely that the expression of CD44, VLA-4 and LFA-1 by the lymphocytes in the heart lesions of patients with chronic chagasic cardyomyopathy contributes to the progression of the local inflammatory reaction.

In previous studies we have demonstrated the persistance of DNA sequences of *Trypanosoma cruzi* in heart tissues of autopsied patients who died of chronic chagasic cardiomyopathy and had cardiac inflammation. We subsequently showed that these inflammatory infiltrates were composed predominantly of granzyme A+ cytotoxic CD8+ T cells. This study describes the distribution of HLA antigens and adhesion molecules in these tissues. Up-regulation of HLA class I molecules on myocardial cells may be important in their becoming targets for cell mediated cytotoxicity. Expression of ELAM and up-regulation of HLA DR antigens on endothelial cells may facilitate recruitment of antigen-specific and nonspecific cells to inflammatory sites. The expression of the cell surface

adhesion molecules LFA-1, CD44 and VLA-4 by inflammatory lymphoid cells may be of pathogenetic significance in cell interactions leading to cardiac injury. These studies extend earlier observations and point to new avenues for continuing investigation into the pathogenesis of inflammation in cardiac chagasic disease.

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