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TESE DE DOUTORADO

ESTUDOS SOBRE A FIBROSE SEPTAL HEPÁTICA INDUZIDA POR *Capillaria hepatica.* (PATOGÊNESE E EVOLUÇÃO)

MÁRCIA MARIA DE SOUZA

Salvador - Bahia - Brasil
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Estudos Sobre a Fibrose Septal Hepática Induzida por Capillaria hepatica (Patogênese e Evolução)

MÁRCIA MARIA DE SOUZA

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"Aqui estou eu, na praia do oceano da verdade. Apanhei alguns grãos de areia, porém todo o oceano jaz diante de mim, desconhecido".

Isaac Newton

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RESUMO

ESTUDOS SOBRE A FIBROSE SEPTAL HEPÁTICA INDUZIDA POR *Capillaria hepatica*. (PATOGÊNESE E EVOLUÇÃO). **MÁRCIA MARIA DE SOUZA.** Vários modelos experimentais têm sido utilizado na tentativa de melhor se compreender a etiologia e a patogênese da fibrose hepática. Ratos infectados com o helminto *Capiliaria hepatica* desenvolvem regular e rapidamente um processo de fibrose septal que progride rumo a uma morfologia cirrótica. A patogênese e o destino desta fibrose são pouco compreendidos. O objetivo da presente investigação foi acompanhar o desenvolvimento da fibrose septal, desde a formação inicial até mais de um ano. Alterações vasculares, celulares e da matriz extracelular foram investigadas por meio de métodos histológicos, bioquímicos, imunohistoquímicos, de imunofiuorescência e ultraestruturais em 94 ratos Wistar, infectados com 800 ovos embrionados de *C. hepatica*, e biopsiados ou sacrificados em diferentes intervalos de tempo, do $10^{\underline{0}}$ dia de infecção até mais de um ano. No início, o septo exibiu diferentes tipos celulares, especialmente fibroblastos e células endoteliais. As células estreladas hepáticas, miofibroblastos ou céllas desmina-actina positivas foram raras. Os vasos sangüíneos apareceram proeminentes nos septos quando preparações de actina, laminina e colágeno IV foram examinadas. Seis meses após a infecção a quantidade de colágeno diminuiu no septo, o que foi confirmado pela dosagem da hidroxiprolina que foi estatisticamente significante. A proliferação vascular (angiogênese) apareceu não apenas como um achado principal na formação do septo, mas também como um condutor da progressão do septo em direção a outro espaço porta. Essas alterações vasculares e estromais sofreram uma redução considerável em torno do $6^{\underline{0}}$ mês da infecção. Contudo, os septos fibrosos ficaram estáveis no $6^{\underline{0}}$ mês, incorporados à nova estrutura hepática, que resultou da fibrose septal. Nossas observações sobre o modelo experimental de fibrose septal nos permitu concluir que a fibrose septal inicia-se entre o $25^{\underline{0}}$ e $27^{\underline{0}}$ dias após a infecção, principalmente a partir dos espaços porta . Esses septos são formados, predominantemente, por células endoteliais e fibroblastos proliferados com poucas células α-actina positivas. A proliferação endotelial e fibroblástica precoce é acompanhada de aumento de expressão de TGF-β, TGF-βR e PDGF nas células dos septos em formação. Após um ano ou mais de infecção os septos fibrosos permanecem em associação com os vasos que brotaram a partir dos espaços-porta e esses vasos persistem, lançando o sangue nos sinusóides sem causar alteração da função hepática.
Palavras-chave: *Capillaria hepatica.* Fibrose hepática. Angiogênese. Rato.

ABSTRACT

STUDIES ON *CAPILLARIA HEPATICA*-INDUCED HEPATIC SEPTAL FIBROSIS (PATHOGENESIS AND EVOLUTION). **MÁRCIA MARIA DE SOUZA.** Several experimental models have been used in an attempt to better understand the etiology and pathogenesis of hepatic fibrosis. Rats infected with the helminth *Capillaria hepatica* regularly and rapidly develop a process of septal fibrosis that progress toward a cirrhotic morphology. Pathogenesis and fate of this fibrosis are poorly understood. The present investigation aimed at following the development of septal fibrosis, from its early formation up to to more than a year. Vascular, cellular and extracellular matrix changes were investigated by means of histological, biochemical, immunohistochemical, immunofluorescence and ultrastructural methods in 94 Wistar rats, infected with 800 embryonated eggs of *C. hepatica*, and biopsied or sacrificed at different time intervals, from the 10^{th} day following infection up to one year later. At the beginning the septa exhibited different cell types, especially fibroblasts and endothelial cells. Hepatic stellate cells, myofibroblasts or demin-actin-positive cells were rare. Blood vessels appeared prominent in the septa when preparations for actin, laminin, collagen IV were examined. Six months after infection the amount of collagen diminished in the septa, which was confirmed by hydroxyprolin measurements in a statistically significant way. Vascular proliferation (angiogenesis) appeared not only as a major finding in septal formation, but also as a leading change in the infiltrating progression of the septum toward another portal space. These vascular and stromal septal changes underwent a considerable reduction by the 6^{th} month of infection. However, the fibrous septa were stabilized from the 6^{th} month on, blood vessels being incorporated into the new hepatic structure, which resulted from septal fibrosis.

Present observations indicated that *C. hepatica*-induced septal fibrosis, which departed from portal spaces, started around the 25th – 27th day following inoculation. Septa were predominantly composed of endothelial cells and fibroblasts plus a few α-actin cells. The early proliferation of endothelial cells and fibroblasts were accompanied by an augmented expression of TGF-β, TGF-βR and PDGF. After one year or longer, thin fibrous septa remained associated with blood vessels that had sprouted from portal spaces and were seen to conduct portal blood directly into the hepatic sinusoids. Therefore, such septal vessels seemed functionally active, which explain their persistence.

KEY WORDS: *Capillaria hepatica*. Hepatic Fibrosis. Angiogenesis. Rat.

LISTA DE ABREVIATURAS

bFGF – Fator de crescimento do fibroblasto - b

CCl_4 – Tetracloreto de carbono

CEH – Célula estrelada hepática

EGF – Fator de crescimento endotelial

HGF – Fator de crescimento do hepatócito

IFN-γ – Interferon Gama

IGF-1 – Fator de crescimento de insulina - 1

IGF-like - Fator de crescimento semelhante a insulina

MCP-1 – Proteína quimioatraente de nonócito-1

MMP – Metaloproteinase de matriz

PDGF – Fator de crescimento derivado de plaqueta

TGF-β – Fator de Transformação do Crescimento beta

TGF-βR – Receptor do Fator de Transformação do Crescimento beta

TGF-β1 – Fator de Transformação do Crescimento beta - 1

TIMP – Inibidor de Metaloproteinase tecidual

TNF-α – Fator de Necrose Tumoral

VEGF – Fator de crescimento do endotélio vascular

SUMÁRIO

1 INTRODUÇÃO

O modelo de fibrose hepática que aparece associado com a infecção por *Capillaria hepatica* em ratos foi descrito por FERREIRA & ANDRADE (1993), e desde então tem suscitado a todos nós do Laboratório de Patologia Experimental um grande interesse quanto à sua patogenia. A observação preliminar sugeria que, produtos parasitários seqüestrados no interior do fígado liberariam antígenos ou outras moléculas que, lenta e gradualmente, iriam excitar à distância o eixo de células não parenquimatosas, constituído pelas células de Kupffer, de um lado, e pelas células estreladas de Ito, de outro. Estas sugestões se baseavam em observações histológicas seqüenciadas. Posteriormente, SANTOS e colaboradores (2001), demonstraram que os elementos patogenéticos mais importantes derivavam da interação hospedeiro-parasita e que os ovos ou os vermes, isoladamente, não reproduziam o quadro de fibrose septal visto em 100% das infecções experimentais. Recentemente, LEMOS e colaboradores (2002), tentaram tolerizar ratos neonatos aos antígenos de *C. hepatica* e, com antígenos de ovos imaturos, administrados por via oral, conseguiram inibir parcialmente a fibrose septal induzida por uma infecção verdadeira. Estes achados acentuaram a participação de mecanismos imunes na patogenia da fibrose septal associada com a infecção de ratos por *C. hepatica*.

A presente investigação sobre a patogenia da fibrose septal pretende determinar onde e quando a fibrose se inicia, quais são os principais elementos celulares envolvidos na sua gênese, qual o seu potencial evolutivo e o seu significado. Procura-se identificar, seqüencialmente, os elementos celulares e

matriciais, desde os estágios mais iniciais até a formação definitiva dos septos. Para tal, foi determinado o momento preciso da infecção em que a formação dos septos se iniciava. Este período foi identificado dentro de uma faixa de dez dias, após os animais infectados terem sido sacrificados e examinados diariamente. Em complementação, animais com infecções tardias, alguns com mais de um ano da inoculação, foram incluídos, pois as alterações vasculares encontradas foram fundamentais para o entendimento do significado da fibrose septal. O material disponível foi estudado com técnicas histológicas, de microscopia eletrônica de transmissão, de imuno-histoquímica, de injeções vasculares de massas coradas e de injeções de plástico seguidas de corrosão em ácido forte, de dosagens bioquímicas de colágenos e de técnicas sorológicas.

Como conseqüência, dois aspectos conceituais originais emergiram: um relacionado ao papel fundamental que a angiogênese mostrou desempenhar na patogenia deste tipo de fibrose hepática; e o outro, sobre o papel das alterações vasculares na manutenção ou irreversibilidade desta mesma fibrose.

1.1 A FIBROSE SEPTAL INDUZIDA POR SORO DE PORCO

A fibrose septal é bem reproduzida no modelo experimental em que ratos são injetados com soro total de porco duas vezes por semana, por 16 a 20 semanas, por via intraperitoneal. Ao fim das injeções, 50 a 75% dos animais desenvolvem uma fibrose septal, que pode se acentuar e chegar a configurar uma cirrose quando os septos acabam por delimitar nódulos hepatocitários por todo o fígado (PARONETTO & POPPER 1966, ANDRADE 1991). Esta fibrose

parece se iniciar na zona perisinusoidal, onde as células estreladas de Ito proliferam e secretam um excesso de matriz extracelular. O elemento do soro que desencadeia o processo está ligado à sua fração albumina. A via de administração do soro também tem importância, pois quando se substitui à via peritoneal pela subcutânea a fibrose septal hepática não se forma (ANDRADE & GODOY, 1996). É possível que o material injetado no peritôneo entre em contato mais prontamente com as células de Kupffer, local onde as técnicas de imunofiuorescência têm demonstrado proteínas do soro de porco no seu citoplasma. Destas células partem, provavelmente, estímulos através de citocinas, como o TGFβ, que induzem as variadas alterações moleculares nas células estreladas de Ito. Sugere-se que a princípio ocorra uma ação das metaloproteinases e que a digestão da pequena quantidade de matriz existente nos espaços de Disse seja o sinal para o início da síntese da matriz extracelular em maior escala. A persistência de fibrose na zona III, uma vez que o estímulo se dá em todos os sinusóides, ainda não está bem esclarecida, mas supõe-se que a baixa concentração de oxigênio nesta zona provavelmente interfira com os processos de degradação e reabsorção do colágeno, favorecendo a sua acumulação.

A fibrose septal induzida pela administração de soro heterólogo, diferencia-se de outros tipos de fibrose hepática e cirrose induzidas por substâncias químicas, tais como CCl_4 ou D-galactosamina, e não está associada com evidências morfológicas de regeneração, nem com síntese aumentada de DNA pelas células parenquimais (RUBIN et al., 1968; SENOO & WAKE, 1985). Dois fatos fundamentais decorrem da observação deste modelo: o primeiro é que a fibrogênese hepática pode se desencadear na ausência de

inflamação crônica evidente e/ou de necrose hépato-celular; o segundo, é que o processo tem uma base imunológica. Em trabalho recente, BHUNCHET e colaboradores (1996), observaram que 15 ratos Wistar tratados com soro de porco duas vezes por semana, a partir do primeiro dia pós-natal e até 18 semanas mais tarde, ficaram imunologicamente tolerantes às proteínas do soro estranho. Desta maneira, eles não desenvolveram fibrose, nem anticorpos contra as proteínas do soro de porco quando repetidamente injetados. Por outro lado, 16 ratos Wistar tratados a partir da 8ª semana de vida, e até 10 semanas mais tarde, desenvolveram fibrose em 75% (12/16). Também foi demonstrado que, nos ratos do primeiro grupo, não havia interferência com a capacidade geral de formar tecido fibroso, pois eles puderam desenvolver fibrose quando tratados com tetracloreto de carbono (CCl_4)durante quatro semanas.

1.2 A FIBROSE SEPTAL INDUZIDA POR *Capillaria hepatica*

FERREIRA & ANDRADE (1993), verificaram que ratos infectados com o helminto *Capillaria hepatica* desenvolviam fibrose septal do fígado. Este helminto tem uma distribuição cosmopolita e é muito comum nos ratos de esgoto (*Rattus rattus* e *Rattus norvegicus*). Caracteriza-se por se desenvolver no interior do fígado do hospedeiro, aonde vêm a morrer pouco depois de atingir a maturidade, sendo seus ovos retidos, vindo a ser liberados para o exterior somente após a morte e desintegração do hospedeiro. A morte do verme no interior do fígado provoca uma reação necro-inflamatória focal, que logo é circundada por uma cápsula fibrosa. No interior da cápsula os detritos

dos vermes mortos e os ovos são paulatinamente degradados na presença de uma reação inflamatória granulomatosa, com tendência fibrosante. O curioso é que, justamente quando as lesões inflamatórias focais mostram evidências de involução, começam a aparecer os septos fibrosos, não só em torno das lesões parasitárias focais, mas, ao mesmo tempo, em várias outras áreas do fígado, sem aparente continuidade direta com as lesões parasitárias. Pouco a pouco, todo o fígado fica septado pelos tratos fibrosos, que tendem a delimitar porções nodulares no parênquima hepático. Durante o tempo de encapsulamento, degradação e substituição fibrosa acima citados, os antígenos derivados dos vermes persistem, o que pode ser demonstrado através de reação de imunofluorescência (FERREIRA & ANDRADE, 1993). Provavelmente, uma situação semelhante acontece com a esquistossomose e a fasciolíase, em vários hospedeiros, inclusive no homem, onde vermes podem morrer e ser seqüestrados no interior do fígado e onde o processo de fibrose septal também aparece, por vezes de maneira proeminente (ANDRADE, 1988; DARGIE et al., 1974).

O modelo de fibrose septal da capilaríase hepática parece ainda mais interessante para os estudos sobre patogenia e quimloterapia da fibrose do que aquele induzido pelo soro de porco. No primeiro modelo a fibrose se desenvolve em quase 100% dos animais, a partir de infecções leves ou moderadas, em torno dos 40 dias da inoculação, enquanto com o soro de porco as melhores possibilidades ficam em torno de 60-70% dos casos. Este fato tem fundamental importância quando se procura testar uma droga anti-fibrosante, por exemplo.

1.3 FIBROGÊNESE HEPÁTICA

O termo fibrose é usado para definir a acumulação excessiva de tecido conjuntivo (colágeno) no parênquima dos órgãos (POLI, 2000). No fígado, o acúmulo progressivo de tecido conjuntivo, representa um evento freqüente que ocorre em resposta a agressão persistente ou crônica. A fibrose hepática é caracterizada pela deposição aumentada e alteração na composição da matriz extracelular, com um excesso de colágenos dos tipos I, III, IV, V e VII. Quando avançada, a arquitetura do fígado é distorcida por densas faixas de colágenos, que criam estruturas vasculares e envolvem ilhas de células parenquimais em regeneração; essas alterações são características da cirrose (IREDALE et al., 1998).

As evidências acumuladas indicam que a fibrose hepática é dinâmica e pode ser bidirecional (envolvendo fases de progressão e regressão) e que, em adição à síntese aumentada de matriz, este processo patológico envolve maiores alterações na regulação da degradação da matriz (ARTHUR, 2000; BENYON & ARTHUR, 2001). Alguns estudos têm mostrado que, se a causa original da lesão hepática é removida, a regressão da fibrose e da cirrose hepática pode ocorrer em doenças hepáticas mesmo em fases avançadas (SCHIFF et al., 2000; HAMMEL et al., 2001).

A fibrogênese é um processo complexo em que as citocinas e os fatores de crescimento localmente produzidos agem em vários tipos celulares. As citocinas modulam tanto as respostas proliferativas quanto a produção de matriz. Algumas das citocinas conhecidas por induzir as proteínas de fase aguda são localmente liberadas durante o desenvolvimento da fibrose hepática.

Contudo, não está claro se o espectro completo das proteínas de fase aguda é induzido e que papel essas proteínas teriam no processo de fibrose (MOSHAGE, 1997).

O processo fibrótico envolve vários tipos celulares e diferentes fatores de crescimento que promovem uma excessiva fibrogênese, com deposição exagerada de matriz extracelular (MEC). As células de Kupffer, os leucócitos exudados e as células estreladas de Ito são os elementos chave da fibrose hepática (POLI, 2000). Dentre esses eventos, dois eventos são de maior importância no estabelecimento da fibrose hepática: a ativação das células de Ito e a expressão aumentada do fator de transformação do crescimento-beta (TGF-β) [NAKATSUKASA et al., 1990; PINZANI et al., 1989; POLI, 2000; ROCKEY & CHUNG, 1996; WEINER et al., 1990]. O TGF-β é expresso por muitos tipos celulares incluindo as células de Ito ativadas e as células de Kupffer (MOSHAGE, 1997). No curso da agressão hepática crônica, o TGF-β tem um proeminente papel na estimulação da fibrogênese através da ativação de células semelhantes a miofibroblastos derivadas de células de Ito (TAHASHI et al., 2002). Ao lado do TGF-β, o fator de crescimento derivado de plaquetas (PDGF), e certos constituintes da matriz extracelular, tal como fibronectina EIII-A estimulam as células de Ito e/ou a fibrogênese (FRIEDMAN, 1993; MATSUOKA et al., 1989). Em contraste, IFN-γ e certos elementos da matriz são inibitórios (De LEEUW et al., 1984; FRIEDMAN et al., 1989). Na presença de alteração tecidual hepática crônica/inflamação, esses e outros diferentes fatores solúveis, pró e anti-fibrogênicos, estão simultaneamente ativos no tecido. Nenhum desses fatores trabalha isoladamente, havendo uma complexa interação em uma rede desses mediadores, seus alvos e a MEC. Por essas

razões, os resultados de investigações *in vitro* sobre o efeito de um ou mais agonistas em CEHs em culturas não refletem a complexidade da situação que ocorre *in vivo* (PINZANI et al., 1998).

Os eventos proteolíticos têm um importante papel na iniciação e na perpetuação da fibrose. O TGF-β é secretado como um precursor latente inativo. A ativação do TGF-β latente em um TGF-β ativo é mediada por uma protease, provavelmente pela plasmina produzida pelas células endoteliais. O TGF-β é um potente fator pró-fibrogênico e estimula diretamente as proteínas da matriz enquanto, inibe a degradação da matriz intersticial via inibição da síntese de metaloproteinases de matriz (MMPs) e síntese dos fatores tissulares inibidores de metaloproteinases (TIMPs). As MMPs são sintetizadas como precursores inativos (proMMPs) que são ativadas pela plasmina. O TIMP inibe tanto as MMPs ativas quanto a conversão das proMMPs latentes em MMPs ativas (MOSHAGE,1997). Em doenças hepáticas crônicas e em modelos animais de fibrose hepática, aumentos significantes na expressão de TIMP-1 e TIMP-2 têm sido observados. Estudos com fígados humanos provenientes de hepatectomias (obtidos de pacientes submetidos a transplantes) têm demonstrado expressão aumentada de TIMP-1, TIMP-2 ou ambos em pacientes com colangite esclerosante, atresia biliar, cirrose biliar primária, e hepatite autoimune (BENYON, et al., 1996; IREDALE et al., 1995). Por hibridização *in situ*, transcritos de TIMP-1 e TIMP-2 têm sido localizados predominantemente em CEHs em fígado humano fibrótico e em modelos animais de fibrose hepática (HERBST et al., 1997). O TIMP-1 inibe a apoptose de linfócitos B e de células epiteliais. (GUEDEZ et al., 1998; LI et al., 1999).

Várias evidências indicam que as células estreladas de Ito são as células centrais para o processo de fibrose, como as maiores produtoras de proteínas fibrilares e não fibrilares da matriz (IREDALE et al., 1998). Elas são usualmente células quiescentes mas, em resposta ao estímulo, sofrem um processo de ativação, após o que se tornam proliferativas e sintetizadoras de matriz extracelular rica em colágeno tipo I. A iniciação da ativação de tais células se deve à uma estimulação parácrina. Contudo, estímulos autócrinos e parácrinos são necessários para a sua perpetuação, o que promove várias mudanças funcionais nestas células (REEVES & FRIEDMAN, 2002). Elas mostram características comuns às das células do músculo liso e miofibroblastos. Em cultura primária mostram reações de coloração positivas para os filamentos intermediários, vimentina e desmina (De LEEUW et al.,1984; TSUTSUMI et al., 1987). No fígado normal, as CEHs no espaço de Disse são mantidas num estado quiescente, fenótipo não fibrogênico, em parte porque elas estão em contato com uma complexa matriz extracelular formada pelo colágeno de tipo IV, laminina e proteoglicanos. Quando removida deste ambiente, elas são ativadas para um fenótipo miofibroblástico pró-fibrogênico, e perdem suas gotículas de gordura intracelulares, constituídas por retinóides, típico do que é visto num fígado fibrótico (ARTHUR, 2000; OKUNO et al., 1999). As células estreladas ativadas expressam α-actina de músculo liso (α-SMA) e pró-colágeno-I e são conhecidas por serem as maiores produtoras de colágenos e outras proteínas da matriz que são depositadas durante a fibrose hepática (IREDALE et al., 1998).

As células estreladas compreendem 15% do número total de células hepáticas residentes. No fígado normal elas são as principais armazenadoras

de retinóides (vitamina A). Tais células constituem um grupo heterogêneo de células que são funcional e anatomicamente similares mas diferentes na sua expressão de filamentos do citoesqueleto, no seu conteúdo de retinóide, e no seu potencial para produção da matriz extracelular. O estímulo que inicia a ativação da célula estrelada é derivado da resposta à agressão aos hepatócitos, às células endoteliais vizinhas e células de Kupffer, e por rápida e súbita alteração induzida na composiçao da matriz extracelular (FRIEDMAN, 2000). Em trabalho recente, SAFADI & FRIEDMAN (2002) afirmaram que todos os tipos celulares vizinhos às células estreladas, incluindo entre estes as plaquetas e os leucócitos, participam como estimuladores nas fases iniciais das mudanças fenotípicas sofridas por estas células. Acrescentaram ainda, que as células endoteliais têm um duplo papel na ativação inicial das células estreladas. A agressão às células endoteliais estimula a produção de um fragmento variante de fibronectina celular (isoforma EIIIA), que tem um efeito ativador sobre as células estreladas. Adicionalmente, as células endoteliais convertem o TGF-β1 latente para a forma fibrogênica (ativa) através da ativação da plasmina (FRIEDMAN, 2000). Os hepatócitos e as células de Kupffer são também potentes geradores de produtos intermediários reativos de derivados de oxigênio. Esses compostos exercem estimulação parácrina nas células estreladas (FRIEDMAN, 2000). No processo de ativação das células de Ito, a infiltração e a ativação das células de Kupffer exercem um proeminente papel, coincidindo o influxo das células de Kupffer com o aparecimento dos marcadores de ativação daquelas células. As células de Kupffer podem estimular a síntese de matriz, a proliferação e a liberação de retinóides pelas

células estreladas através da ação de citocinas, especialmente, do TGF-β1 (SAFADI & FRIEDMAN, 2002).

A perpetuação da ativação das células estreladas envolve respostas fenotípicas chaves mediadas pelo efeito aumentado de citocinas e remodelamento da matriz extracelular. As respostas aumentadas às citocinas ocorrem através de múltiplos mecanismos incluindo expressão aumentada de receptores de membrana e o aumento na expressão de moléculas transdutoras de sinais. (FRIEDMAN, 2000).

O continuado remodelamento da matriz extracelular durante a fibrogênese afeta todas as respostas celulares, caracterizando a progressiva agressão hepática. A matriz subendotelial de baixa densidade é progressivamente substituída por uma matriz rica em fibrilas de colágeno. Esta mudança fundamental na composição da matriz extracelular afeta o comportamento dos hepatócitos, do endotélio e das células estreladas. A formação de fibrilas da matriz extracelular também acelera a ativação das células estreladas (FRIEDMAN, 2000).

Quando o fígado é agredido, as células estreladas sofrem uma alteração fenotípica. Essa agressão leva a ruptura do padrão da matriz extracelular normal e a exposição das células estreladas quiescentes a citocinas, fatores de crescimento e produtos do estresse lipoperoxidativo que promovem a sua transdiferenciação para um estado ativado (PINZANI et al., 1998). Isto inclui: proliferação, contractilidade, fibrogênese, degradação da matriz extracelular, quimiotaxia, perda de retinóides, liberação de citocinas e quimioatração por leucócitos (FRIEDMAN, 2000; TAMATANI et al., 1998). Juntas, essas modificações originam um tipo celular com acentuadas

propriedades proliferativas, sintéticas e contráteis. As evidências têm mostrado que existe uma população bem diversa de células mesenquimais, tanto no fígado normal, quanto no agredido, com diferentes conteúdos de vitamina A e expressão variável de filamentos intermediários característicos de células miogênicas e/ou neurais. Em particular, uma crescente lista de marcadores neurais tem sido identificada em células estreladas, incluindo Rho N (GEERTS, 2001; NISHI et al., 1999), proteína acídica fibrilar glial, nestina e receptores de neurotrofina. Como resultado desta heterogeneidade fica a dúvida se todas as células mesenquimais hepáticas têm a mesma origem embrionária e se as células estreladas formam uma população homogênea. Desta forma, surge uma importante questão sobre se todas as células de Ito têm a mesma capacidade de sofrer ativação ou apoptose (ENG & FRIEDMAN, 2000).

Ainda que as células estreladas ativadas (semelhantes aos miofibroblastos) sejam conhecidas como o principal tipo celular fibroblástico envolvido na fibrogênese hepática, outros tipos celulares da linhagem fibroblástica tais como os fibroblastos portais ou miofibroblastos vasculares (pericitos) podem também ter um potencial fibrogênico, como sugerido por estudos *in situ* (BALLARDINI et al.,1994; SCHMITT-GRAFF et al., 1993).

1.4 A *Capillaria hepatica*

A *C. hepatica* é um nematódeo tricuroídeo muito comum como parasito do rato e de outros roedores, tendo sido encontrado em numerosas espécies de mamíferos, como o esquilo, o porco-do-mato, a lebre, o cão, o gato e o macaco e mesmo o homem (MOURA, 1991).

A fêmea mede 2cm de comprimento e o macho apenas a metade. O verme adulto (*C. hepatica*) tem simetria bilateral, corpo cilíndrico, alongado, não segmentado, revestido por cutícula e desprovido de membros articulados. Possui um aparelho digestivo completo e cavidade geral sem revestimento epitelial. Apresenta também dimorfismo sexual (WRIGHT, 1961). Os ovos são muito parecidos com os do tricocéfalo, com as mesmas cascas e rolhas polares, porém a membrana mais externa é atravessada por minúsculos canais que lhe emprestam aspecto estriado muito característico (McQUOWM, 1954). Medem 50 a 69μm de comprimento por cerca de 30μm de largura (MOURA, 1991) . A infecção verdadeira ocorre com a ingestão pelo hospedeiro de ovos embrionados que eclodem no ceco, atravessam a mucosa, liberando larvas que migram pelo sistema porta para o fígado. Os vermes permanecem no fígado onde morrem pouco depois e liberam os seus ovos (FARHANG-AZAD et al., 1977). Os vermes em desintegração e os ovos liberados no parênquima hepático provocam reação inflamatória granulomatosa, proliferação de fibroblastos acompanhada de fibrose focal. A reação forma uma cápsula em torno dos ovos e dos vermes desintegrados (LUTTERMOSER, 1938). Os ovos só amadurecem fora do fígado, quando após a morte do hospedeiro e desintegração dos tecidos eles são liberados no meio ambiente. Portanto, a *C. hepatica* pertence ao grupo dos geo-helmintos, parasitos transmitidos a partir do solo (MOURA,1991).

A taxa de infecção dos ratos (*Rattus novergicus*) é da ordem de 40 a 90%, em certas áreas (MOURA, 1991). CHIEFFI et al., (1981), necropsiaram 205 (191 *Rattus novergicus* e 14 *Rattus rattus*) murídeos capturados vivos no

município de São Paulo e verificaram que destes, 120 (59%) estavam parasitados por *Capiliaria hepatica*.

A *C. hepatica* é um helminto de elevada prevalência em roedores domésticos e, excepcionalmente, afeta também o homem.

Segundo GALVÃO (1976), muitos casos humanos não fatais passam despercebidos, uma vez que os ovos dos parasitas não aparecem nas fezes nos casos de infecção verdadeira, sendo a única possibilidade de diagnóstico reservada aos testes imunológicos.

Na tentativa de investigar a presença de anticorpos contra *C. hepatica* no soro de 500 crianças em Salvador, Bahia, GALVÃO (1979) sugeriu, em seu trabalho, que a *C. hepatica* pode se relacionar com o homem em nosso meio, uma vez que, das 500 crianças que tiveram seus soros testados através de reações de imunofluorescência, 9 tiveram um padrão peculiar e intenso, mesmo após elevada diluição do soro, contra estruturas de vermes adultos e seus ovos, representando provavelmente casos subclínicos da infecção verdadeira pela *C. hepatica.* Os demais casos, apresentaram padrões de fluorescência os mais variados, refletindo possivelmente infecções espúrias por *C. hepatica* ou reações cruzadas com antígenos os mais variados. Contudo, devido às baixas condições de higiene e moradia entre a população de baixa renda, existe a possibilidade de que a capilaríase hepática tenha um papel na patologia humana maior do que é comumente admitido (GALVÃO, 1981).

Na Europa foram descritos pelo menos 11 casos de infecção hepática, assim como outra dúzia em diferentes países do mundo, inclusive um no Brasil e outro no México. Eram todos casos gravíssimos e quase todos terminaram com o falecimento do paciente (MOURA, 1991).

O quadro anatomopatológico produzido é o de uma hepatite parasitária, com formação de granulomas ao redor dos acúmulos ovulares. Não há descrição clara de fibrose septal nestes casos. No centro da lesão há, por vezes, focos de necrose. O fígado aumenta de tamanho, chegando a hepatomegalias volumosas e dolorosas (MOURA, 1991). KOHATSU et al., (1995), descreveram um caso de lesão causada por infecção com *C. hepatica,* revelada através de ultrasonografia, no segmento 6 do fígado, em uma mulher de 32 anos de idade, em Okinawa, Japão. O exame de laboratório mostrou leucocitose (10.400/mm^3) com 22% de eosinófilos e leve alteração dos testes de função hepática. O tumor foi removido cirurgicamente e se revelou como uma reação do tipo granulomatosa, com necrose, com infiltração eosinofílica ao redor do nematódeo em desintegração.

Clinicamente, nas infecções graves, há febre alta, anorexia, vômitos, extrema fraqueza e anemia, há leucocitose e eosinofilia intensas, podendo os eosinófilos chegar a 80% das células do sangue. Também se constata hipergamaglobulinemia (BHATTACHARYA et al., 1999; MOURA, 1991).

Os ovos do parasito não aparecem nas fezes dos pacientes. Nos casos acima referidos, o diagnóstico foi estabelecido por biópsia hepática ou necrópsia. Entretanto, os ovos de *C. hepatica* têm sido encontrados nas fezes de pessoas sadias (no Panamá), indicando provavelmente a ingestão de fígado de animais parasitados (caça) e trânsito dos ovos imaturos do helminto pelo intestino, sem eclosão, devido à sua imaturidade (MOURA, 1991).

É possível que a infecção com pequeno número de vermes transcorra com pouca sintomatologia e sem diagnóstico, até a cura espontânea

(MOURA,1991). Supõe-se que os disseminadores de ovos do parasito, no ambiente humano, sejam os cães e gatos que caçam ratos infectados.

1.5 FIBROSE HEPÁTICA E ANGIOGÊNESE

A fibrose septal é um achado comum em várias doenças crônicas do fígado. No modelo da Capilaríase hepática ela é representada por septos fibrosos, longos e finos, que aparecem sulcando o parênquima hepático ao longo da zona III do ácino, conectando espaços porta e veias centrais entre si e, também, veias centrais com espaços porta, muitas vezes terminando como um esporão no interior do parênquima. Uma vez que a fibrose septal se localiza, pelo menos no início de sua formação, nos espaços de Disse e se continua numa localização perisinusoidal, suas relações com as células de Ito são evidentes, mesmo em microscopia ótica.

Embora seja um achado inespecífico, a fibrose septal assume proeminência em algumas entidades da Patologia Humana, como a cirrose septal incompleta (SCIOT, et al., 1988) e a hepatite crônica septal (GERBER & VERNACE, 1974). Estas condições permanecem como sendo de etiologia obscura, o que acentua o interesse pela etiologia e patogênese deste tipo de fibrose. A cirrose septal incompleta foi sugerida como uma nova e importante entidade mórbida do homem (DHAR & CHAWLA, 1995).

MOSCHCOWITZ (1948), afirmava que o desenvolvimento dos septos fibrosos decorrente do tecido mesenquimal do espaço porta era promovido pelo processo de angiogênese, e que as reações inflamatórias induziam à formação de novos capilares, em pontos onde alguns deles colapsavam, o que contribuía para a estimulação da formação de tecido conjuntivo. HARTROFT & RIDOUT

(1951), insistiam que a presença de trabeculações na cirrose experimental causada por deficiência em colina, devia-se à condensação do estroma pré-existente e não á proliferação do tecido conjuntivo. Este conceito, entretanto, foi mais tarde refutado por uma série de investigações conduzidas por POPPER (1970).

A maioria das agressões hepáticas envolve alterações morfológicas e patológicas dos vasos intra-hepáticos (EL-ASSAL et al., 1998; NAKATA et ai., 2002). Quando há redução de microvasos durante a inflamação crônica, necrose prolongada e fibrose, ocorre também a diminuição dos fatores de crescimento relacionados à angiogênese tais como o VEGF, fator de crescimento semelhante a insulina (IGF-like), fator de crescimento de transformação beta (TGF-β) e do fator de crescimento derivado de plaqueta (PDGF) (AKIYOSHI et al., 1998; YAN et al., 1999).

A inflamação e a hipóxia contribuem para a angiogênese em doenças não-neoplásicas, sendo também um forte estímulo para a angiogênese em numerosas desordens. A angiogênese, prolongada e excessiva é uma característica das desordens inflamatórias em muitos órgãos, inclusive no fígado. Monócitos, macrófagos, plaquetas, mastócitos e outros leucócitos liberam miríades de fatores angiogênicos incluindo VEGF, Ang1, bFGF, TGF-β1, PDGF, TNF-α, fator de crescimento do hepatócito (HGF), fator de crescimento semelhante a insulina-1 (IGF-1), proteína quimioatraente de monócito-1(MCP-1), entre muitas outras (PINEDO et al., 1998; SELJELID et al., 1999). Alguns desses fatores atraem células em estado de degeneração que por seu lado liberam fatores angiogênicos adicionais (COUSSENS et al., 1999; SCHAPER et al., 1996;).

A cirrose hepática é caracterizada pela coexistência de necrose, regeneração nodular e septos fibróticos, que levam à insuficiência hepática progressiva e irreversível. A despeito das extensivas investigações funcionais e morfológicas, os mecanismos responsáveis pela falha progressiva do fígado cirrótico são ainda debatidos (MACSWEEN et al., 1987; SCHIFF & SCHIFF, 1993). Alguns autores acreditam que a insuficiência hepática na cirrose é causada pelo desarranjo da estrutura hepática (VAUBOURDOLLE et ai., 1989; MARTINEZ-HERNANDEZ & MARTINEZ, 1991). Sendo assim, o papel principal pode ser atribuído às alterações patológicas da microvasculatura. SCHAFFNER & POPPER (1963), descreveram a presença de uma membrana basal nos sinusóides do fígado cirrótico, sendo esse fenômeno conhecido como capilarização dos sinusóides hepáticos. Subseqüentemente outros trabalhos sugeriram que a modificação dos sinusóides pode contribuir para impedir a troca de nutrientes entre os hepatócitos e o sangue que perfunde o fígado (MARTINEZ-HERNANDEZ, 1984 e 1985).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar a patogênese da fibrose septal hepática induzida por *Capillaria hepatica* em ratos Wistar através de observação sequencial das alterações celulares, vasculares e da matriz extarcelular.

2.2 MANUSCRITO I

Avaliar o papel da angiogênese no processo de fibrogênese hepática causada por *C. hepatica.*

2.2.1 Objetivos Específicos

Acompanhar as alterações hepáticas precoces para tentar identificar os fatores mais relevantes no processo de fibrogênese, através de:

i) caracterização dos tipos celulares envolvidos utilizando microscopia eletrônica e técnicas de imuno-histoquímica;

ii) investigação da expressão de TGF-β, do receptor de TGF- β (TGF-β-R) e do PDGF tentando correlacioná-los com os processos de fibrogênese e angiogênese;

iii) identificação de proliferação vascular septal pela demonstração imuno-histoquímica de Fator de von Willebrand (Fator VIII), Laminina, Colágeno tipo IV e α-actina de músculo liso.

2.3 MANUSCRITO II

Acompanhar a evolução da fibrose septal hepática até um ano após a infecção com *C. hepatica,* na tentativa de esclarecer o seu destino.

2.3.1 Objetivos Específicos

- Avallar o grau de fibrose, comparativamente, através do conteúdo de colágeno em fígados de ratos com 3, 6, 9 e 12 meses pós-infecção com *C. hepatica*;
- Investigar possíveis alterações vasculares hepáticas em animais representativos dos diferentes períodos de infecção acima, através de técnicas histológicas, imuno-histoquímicas e ultraestruturais;
- Estudar a microcirculação hepática através de injeção vascular de fígados de ratos em diferentes momentos da infecção.

3 MANUSCRITO I

Role of angiogenesis in *Capillaria hepatica*-induced septal fibrosis of the liver in rats.

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Background/Aims: Different cell-lines participate in the genesis of septal fibrosis of the liver, but their relative importance has not been established. We studied a new model of *Capillaria hepatica*-induced septal fibrosis, searching for its earliest changes in order to identify the origin and the structures involved and their relative importance.

Methods: Samples of liver tissue taken from the 20^{th} to the 39^{th} day after infection of rats were submitted to routine histology, electron microscopy and immunohistochemistry for the demonstration of SMα-actin, desmin, vimentin, factor VIII, TGF-β and TGF-βR, dendritic cells and PDGF. Extra-cellular matrix elements were investigated by immuno-fluorescence for collagens (types I, III, and IV), laminin and fibronectin. The distribution of septal vessels was studied by injection of India ink into the portal vein system.

Results: Early septa exhibited mononuclear cells, especially fibroblasts and endothelial cells and a few actin-positive fat-storing cells and myofibroblasts. The expression of actin, collagen IV and laminin in the walls of blood vessels, coupled to the demonstration of endothelial cells (factor VIII) revealed the prominent presence of vascular elements

(angiogenesis) during the early process of septum formation. Collagen deposition progressively followed the cellular and vascular changes.

Conclusions: During initiation of *C. hepatica*-induced septal fibrosis, blood vessel proliferation (angiogenesis) from portal areas, together with mesenchymal-cell mobilization, represented an early, prominent, and leading change, sprouting from portal spaces and behaving in a way comparable to bile duct proliferation during biliary obstruction.

Keywords: Hepatic fibrosis, Septal fibrosis, *Capillaria hepatica*, Angiogenesis.

Fibrosis of the liver may develop through different pathways. The one involved with the production of septal fibrosis has aroused much interest because of its peculiarities. Usually, septal fibrosis develops as thin and long fibrous septa, running at the acinar zone III of the hepatic parenchyma, and connecting portal spaces, central veins and portal spaces to central veins. Observations from two main experimental models, the pig-serum model (1, 2, 3) and the *Capillaria hepatica* model (4), have stressed that the fibrosis is not preceded by repeated episodes of hepatocellular necrosis nor overt chronic inflammation. Although septal fibrosis has a parasinusoidal location, the participation of sinusoidal cells, especially the hepatic stellate cells (HSC), which has proved to be so prominent for many types of hepatic fibrosis (5-12), has not been confirmed to be so for the two models above mentioned (4, 13). Actually, septal fibrosis dissects the liver parenchyma, running in between hepatic cell-plates, without causing evident alterations of their normal structure or of the neighboring sinusoids.

Several studies have attempted to investigate the earliest cellular changes of septal fibrosis formation in rats repeatedly injected with pig-serum (3, 14). They disclosed that periportal fibroblasts, and also pericentral ones, play a central role in the initiation and development of the fibrous septum. In the *C. hepatica* model, septal fibrosis regularly occurs in 100% of the rats infected with embryonated eggs, regardless the inoculum size (15). Septal fibrosis develops when the focal necro-exudative lesions caused by the dying worms and their liberated eggs are already being encapsulated and undergoing resorption (4).

By sacrificing infected rats daily, the present study revealed that the earliest signs of septum formation occurred from the 25^{th} up to the 28^{th} day following inoculation. These changes were then analyzed in details, in a sequential study, which included liver samples taken from the 20^{th} up to the 39^{th} day of infection.

Observed changes indicated that the earliest septa appeared as a mixture of mesenchymal cells and blood vessels, sprouting from periportal spaces. The outstanding presence of proliferating blood vessels (angiogenesis) was a constant and impressive feature and so was particularly studied.

MATERIALS AND METHODS

Animals - A total of 40 healthy, outbred Wistar rats, weighing 180-200 g, of both sexes, were used. They were maintained in good housing conditions, with free access to a commercial balanced diet and water. All the animals, except 5 (randomly separated to serve as intact controls), were submitted to infection with 800 mature *C. hepatica* eggs, administered by a gastric tube. These eggs were isolated from the livers of experimentally infected mice, through homogenization in saline followed by repeated washing and

sedimentation. The clean isolated eggs were kept in a humidified Petri dish at 25-28^0 C. during a period of 28 days for embryonation.

Initially, two rats were daily sacrificed, from the 25th up to the 30th day following infection for the sequential study of the lesions. The others, 3 at a time, were submitted to a partial hepatectomy (biopsy), under anesthesia and aseptic conditions, with removal of a liver lobe, on day 25, 29, 32, 36 and 39 following inoculation. Besides the liver fragments removed at necropsy or biopsy, samples of serum were collected at several occasions. Ten previously biopsied animals and five controls were later sacrificed and their portal vein system injected with Indian ink for vascular observations. Two animals at each time-point received an injection of 0.3ml of diluted India-ink into the tail vein, 24 hours before sacrifice for the mapping of Kupffer cells.

Histopathology – Fragments of the liver were fixed for at least 48 hours in buffered (pH 7.2) 10% formalin. Paraffin-embedded sections were stained with hematoxylin and eosin, sirius-red for collagen, PAS-method, with and without diastase digestion, Perls' method for iron, toluidine-blue for intracellular metachromatic granules and orcein for elastic fibers.

Immunofluorescence - Fragments of liver tissue were immediately embedded in Tissue–teck (OCT compound-Miles Inc. Diagnostic Division, Elkhart, USA) and immersed into liquid nitrogen for a few minutes and them kept frozen at -70^0 C in airtight boxes, until the moment they were sectioned in a cryostat at -20^0 C. The sections were submitted to the indirect immunofluorescence technique for the demonstration of collagen isotypes (I,III and IV), laminin and fibronectin. The specific anti-sera were anti-human, polyclonal, obtained in rabbits (Institute Pasteur, France). They were used in dilution varying from 1:40 to 1:100. Details concerning their preparation and tests of specificity appear elsewhere (16). Secondary fluoresceinated anti-rabbit-IgG was obtained from SIGMA, USA.

Immunohistochemistry – For the demonstration of factor VIII, dendritic cells and ED3, fragments of the liver were immediately embedded in Tissue–teck (OCT compound-Miles Inc. Diagnostic Division, Elkhart, USA), frozen into liquid nitrogen, and cryopreserved in a freezer at –70°C until the moment of sectioning.

Sections of 6μm were obtained in a cryostat at –20°C were mounted on slides previously treated with Poly-L-Lisin at 10% (Sigma St. Louis, Mo. USA), fixed in dehydrated Acetone, treated with PBS containing 0.1% Saponin (Sigma St. Louis, Mo. USA) and 1% Bovin Serum Albumin (BSA) (Sigma St. Louis, Mo. USA). For blockade of non-specific ligations sections were treated with BSA 30% - in PBS for 20 minutes at room temperature.

For the demonstration of SM-α actin, TGF-β, TGF-β-R, desmin, vimentin and PDGF, paraffin sections of formalin-fixed tissue were used. Antigen retrieval was accomplished through micro-wave treatment in citrate buffer at pH 6.0.

Cryostat and paraffin sections were incubated with the primary antibodies (Table 1) overnight, at 4°C in an humidified chamber. Primary antibodies were diluted in 2% BSA in PBS (pH 7.4). After, 20 minutes washing into PBS and no fat milk , for a blockade of non-specific ligations. The slides were then incubated with the Kit Peroxidase conjugate to anti-mouse and anti-rabbit immunoglobulins (Dako envision system – labelled polymer K1392). Blockade of the endogenous peroxidase was done with 0.3% H_2O_2 for 30 minutes, at room temperature. The color was developed with 0.06% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, Mo-USA) and 0.06% H_2O_2 plus 1% dimethylsulphoxide (Sigma, St. Louis, Mo, USA). Sections were counterstained with 1%

methyl-green for 2 minutes dehydrated and mounted with Permount. Control sections in which primary antibody was either omitted or replaced by normal rat serum, were used.

Transmission Electron Microscopy – Small pieces of liver (about 1 mm^3) were immediately fixed by immersion into 4% glutaraldehyde in 0.2M cacodylate buffer, pH 7.4, for 1 hr at 4°C, washed in buffer and postfixed with 1% osmium tetroxide, dehydrated in graded concentrations of acetone and embedded in Polybed 812 (Embedding Media Polysciences, INC) Selected ultrathin sections (50-70 nm) were mounted on uncoated copper grids, contrasted with uranyl acetate and a lead citrate. Specimens were examined in a Zeiss EM-9 electron microscope, which was operated at an acceleration voltage of 50 kV.

RESULTS

All infected animals presented several *C. hepatica*-induced focal lesions in their livers. These were encapsulated exudative and granulomatous lesions centered by a necrotic area, containing worm debris and clusters of immature eggs. From these areas, fibrous-cellular septa radiated toward the hepatic parenchyma. But, the earliest signs of septum formation were searched in areas remote from the parasitic lesions. Not all animals developed septal fibrosis at the same time, nor to the same degree. For the group of animals sacrificed daily, no septal fibrosis was observed in the days 20-23, in spite of the presence of severe parasitic lesions. From then on, septal fibrosis became a constant feature. Septum initially sprouted from portal spaces, giving them a stellate appearance. These early septa were thin, rich in vessels and mononuclear cells, and poor in extracellular matrix. With time, the septa connected neighboring portal spaces, sometimes creating a polyhedral structure, leaving a vein at the center, reproducing the structure of the liver of a pig. (Fig. 1A). The presence of collagen was very scarce during the initial days, but it gradually increased with time, while

the number of inflammatory cells diminished. Both collagen isotypes, I and III, were identified from the start (Fig. 4A, B). The septum, both at its cellular and fibrous stages, was limited by normal-looking parenchyma on both sides. Its presence was not associated with neighboring perisinusoidal fibrosis or any other changes evident by light or electron microscopy. The distribution of Kupffer cells, evaluated by immunohistochemistry (anti-ED3) and by *in vivo* staining with Indian ink, did not differ from the normal (Fig. 2E, 1C). No accumulation of Kupffer cells was noted along the septa.

Cells – The combining study with routine histology, electron microscopy and immunohistochemisatry revealed that the numerous cells that formed a linear accumulation along the early septum were predominantly mononuclear and polimorph, with a sparkling of a few polymorphonuclear eosinophils (Fig. 5A). Endothelial cells and fibroblassts were the predominanting cells, recognized by the factor VIII and vimentin markers, respectively (Fig. 2A, 2D) and by ultrastructural characteristics (Fig. 5A, C, D). Desmin and alfa-actin-positive cells appeared in a proportion of approximately 1 to 5 of either fibroblasts or endothelial cells. Fusiform cells, with well-develop endoplasmic reticulum and the presence of myofibrils and a halo of basement membrane-like material (myofibroblasts) was only rarely found. The same for fibroblast-like cells containing fat droplets in the cytoplasm (SHC) (Fig. 5B). The presence of mast-cells, identified by the presence of toluidine-positive metachromatic granules, was not remarkable. These cells were noted partially degranulated under the electron microscope. Hepatic cells situated at the margins of a septum sometimes underwent apoptosis and were incorporated to the other cells present. They then appeared as rounded eosinophilic bodies, with or without a picnotic nucleus, containing diastase-resistant-PAS positive material and variable amount of iron. (Fig. 1D).

Growth Factors – Treatment of the section with anti-TGF-β resulted in a selective staining of the septa (Fig. 3C), which appeared more marked in earlier infections. The marker for its receptor (TGF-β-R) was detected in at least 50% of the cells present in the septa (Fig. 3D). Around 90% of the cells infiltrating the early septa appeared positive for PDGF (Fig. 2F).

Blood vessels – Numerous capillaries, venules and arterioles were present during the formation of the earliest septa from portal spaces. When a septum was fully formed (around the 39th day), the amount of blood vessels was not so conspicuous. The vessels were mainly represented by arterioles and venules. Injection of India ink into the portal system revealed straight vessels running within the septa. Some were appeared isolated, without collaterals for some distance, but terminating into radiating vessels which connected directly with sinusoids. Preparations for the demonstration of α-actin, laminin, and collagen IV (Figs. 3A, B; 4C, D, E) revealed components in the blood vessel walls, such as smooth muscle and basement membrane. Blood vessels appeared prominent, especially in very early septa. Sometimes the vessels were seen at the advancing tip of a septum, giving the impression of leading the way in the dissecting of the parenchyma (Fig. 1E, F).

DISCUSSION

When looking at the full-blown picture of septal fibrosis of the liver in *C. hepatica*-infected rats, one may get the impression that it resulted from a parasinusoidal process, an exaggeration of the normal deposition of collagen fibers by HSC within the Disse space. The observation that neighboring sinusoids maintain their normal appearance turns this possibility unlikely, since it would assume the illogical selection of a particular sinusoid.

It is more evident that the fibrous septum is actually dissecting the hepatic parenchyma and so has departed from somewhere. The observation of an abundant material, representative of the earliest stages of septum formation in *C. hepatica*-infected rats, leaves no doubts that the septa start as buds from the periportal connective tissue. This statement does not mean that other sites are excluded. In the present study, some septa were seen connected to focal parasitic lesions, central veins and to the external liver capsule, but the majority were connected to periportal areas. From this latter, the septa appeared initially sprouting toward another portal space, sometimes mimicking the pig-liver histology. This interportal connections seem evolutively determined, since not only the pig, but other species, such as wild pig, camel, dromedary, racoon, polar bear, and some other mammals, also exhibit a normal periportal fibrous connections.

From the very beginning of septum formation, two elements appeared prominent: blood vessels and cells. Endothelial-cell proliferation and newly formed capillaries are present, but the outstanding morphological feature of the new septum is rather the presence of venules and arterioles. The role of angiogenesis in the pathogenesis of liver cirrhosis has long been stressed. (17). Rappaport et al (18) stated that sprouting of vascular branches, especially of arterioles, takes the leading role in the development of mature scars, i.e. of *fibro-vascular membranes.* Recently, Rosmorduc et al (19) provided evidence for the induction of angiogenic factors, vascular endothelium growth factor (VEGF) and fibroblast growth factor (FGF-2), the most potent angiogenic factors identified thus far, during experimental liver fibrogenesis in rats with bile-duct ligation. They found that VEGF and angiogenesis preceded the onset of cirrhotic lesions. Tuckweber et al (20) discussed that much attention has been given to the hepatic stellate cell (HSC) as the source of the extracellular matrix proteins. However, in the bile duct ligation model, mesenchymal cells

other than HSC may be involved in the early stages of fibrosis development. The periportal fibroblasts may play a dominant role in the early portal fibrosis after bile duct ligation. Many experimental and clinical studies have documented bile duct proliferation as the initial morphologic manifestation of liver lesion after extra hepatic bile duct obstruction. In present study, periportal vascular proliferation, especially observed when actin, laminin and collagen IV were stained, strongly reminded the bile duct situation in the bile-duct ligation model. In the *C. hepatica* model septum formation presents no apparent involvement of bile duct. The model therefore correspond to a bile-duct ligation model where bile ducts appear replaced by blood vessel proliferation.

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Table 1: Antibodies and their sources used in this study

Antibody	Dilution	Source
Mouse anti-TGFR1-β	1:20	NOVO CASTRA
Mouse anti- smooth muscle actin (clone 1A4)	1:100	DAKO
Anti-vimentin (anti-human - clone VIM 3B4)	1:25	DAKO
Mouse anti-TGF beta (anti-human – clone TGFB17)	1:80	NOVO CASTRA
Rabbit anti- PDGF (policlonal, clone AB-1)	1:10	CALBIOCHEM
Anti- desmin (Clone D33)	1:10	DAKO
Mouse anti-macrophages (anti-rat clone ED3)	1:80	BIOSOURCE INTERNATIONAL
Anti- Fator VIII (mouse anti-human)	1:100	DAKOPATTS
Mouse McAb anti- dendritic cells (clone OX-6)	1:80	SERA-LAB

Fig. 1 – **A**: The liver parenchyma is dissected by thin and long fibrous septa, which connects portal space to portal space; H & E, 100X. **B**: The cellularity of the early fibrous septum is evident, with fusiform, fibroblast-like cells predominating; H & E, 200X.

C: Thin early septa appear crisscrossing the hepatic parenchyma. The disseminated, homogeneously distributed tiny black dots represent Kupffer cells, that concentrated Indian-ink pigment into their cytoplasm. Sirius-red staining for collagen, 100X; **D**: Blue, iron-stained macrophages appear among the cells in an early septum, 29 days after *C. hepatica* infection. Perls' method for iron, 400X. **E**: sprouting of blood vessels from a portal space, representing the beginning of septum formation; **F**: the ink appears inside septal vessels which freely communicate with and perfuse the hepatic sinusoids. Portal vein was perfused with Indian ink in a rat with a 29 day-old *C. hepatica*-infection. Counterstained with H & E, 200X.

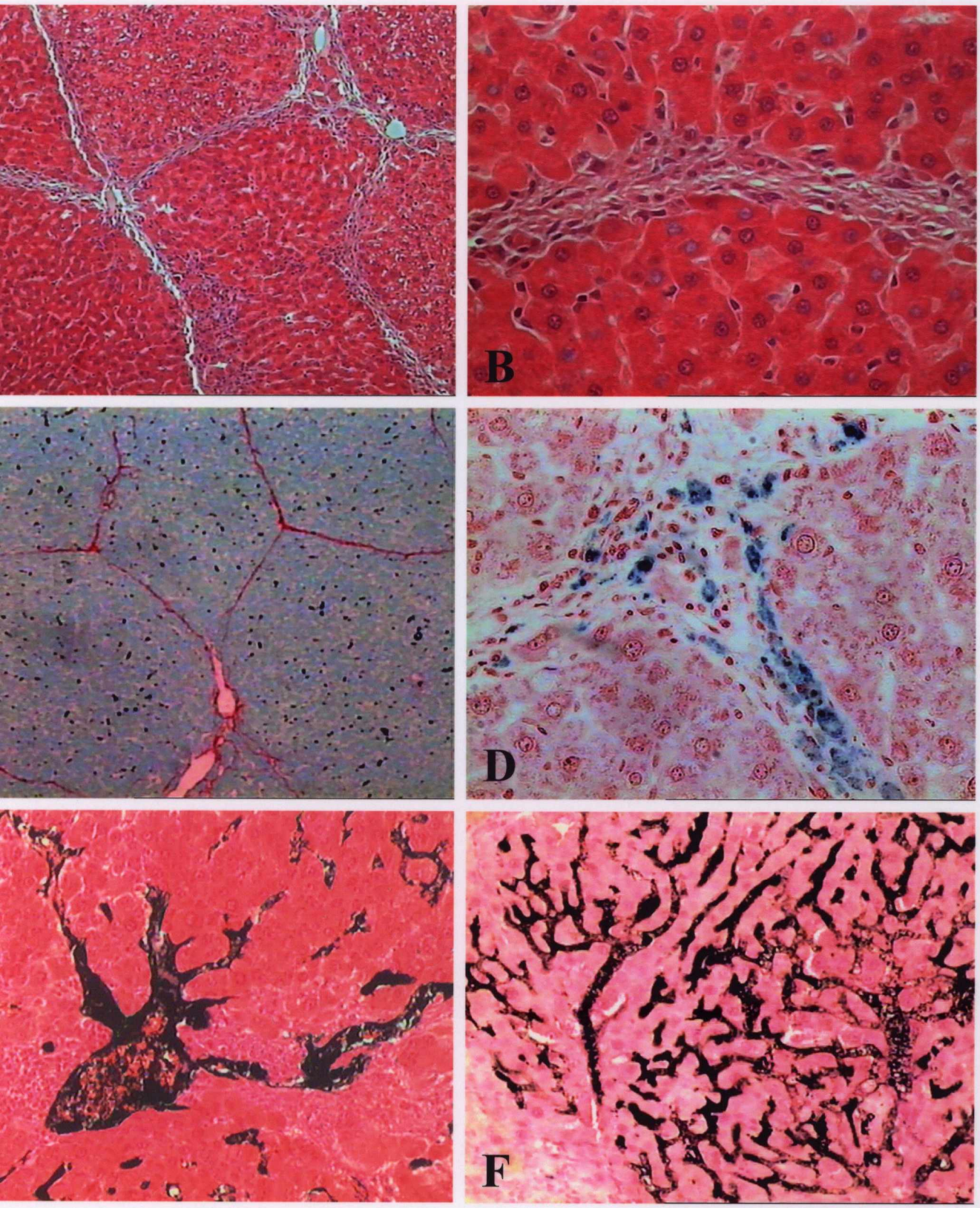

Fig. 1

Fig. 2 – Representative of the phenotypic types of cells present in the liver of rats with early *C. hepatica*-infectin (26-29 days of infection). **A**: Factor VIII; **B**: Dendritric Cells; **C**: Desmin; **D**: Vimentin; **E**: ED3 (macrophages) and **F**: PDGF. There are few cells expressing desmin, and many expressing vimentin, PDGF and Factor VIII. Dendritic cells and macrophages (ED3) have an ubiquitous distribution. Immunohistochemical stains, 200X.for all, except for F (PDGF) =100X.

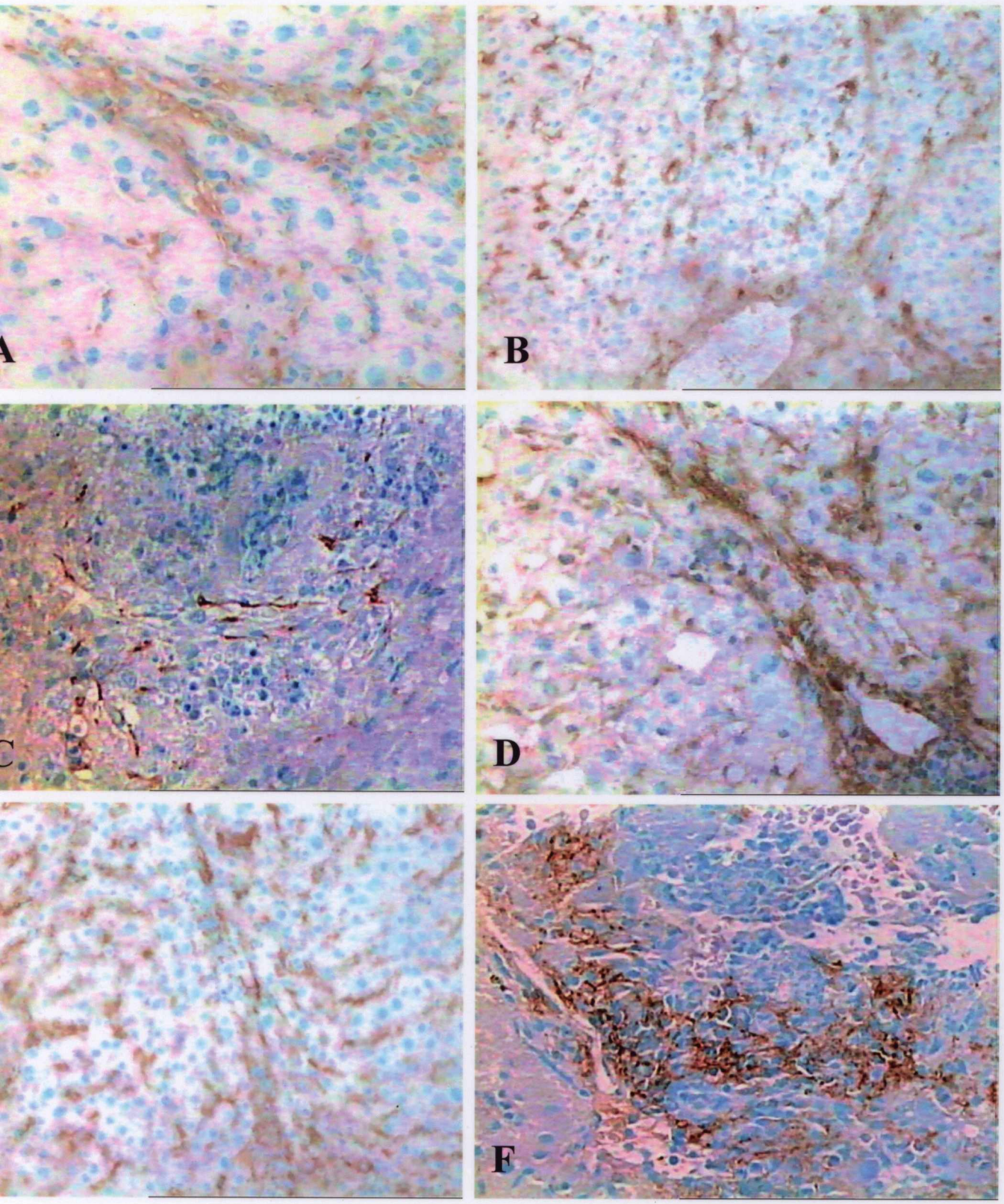

Fig. 2

Fig 3 – **A** and **B**: Actin marks the walls of blood vessels and also a few isolated stellate-cells (arrows) present within the septa formed in the liver of rats with early *C. hepatica*-infection. Imunohistochemistry for SM α-Actin, A = 100X; B = 400X. **C**: The presence of TGF-β is revealed by a selective and diffuse staining of the fibrous septa. **D**: The receptor for TGF-β (TGF-β-R) is expressed by many cells present in portal space (arrows) and septum (arrow heads). (All illustrations from 26-29-day old infection) C = 100X; D = 200X

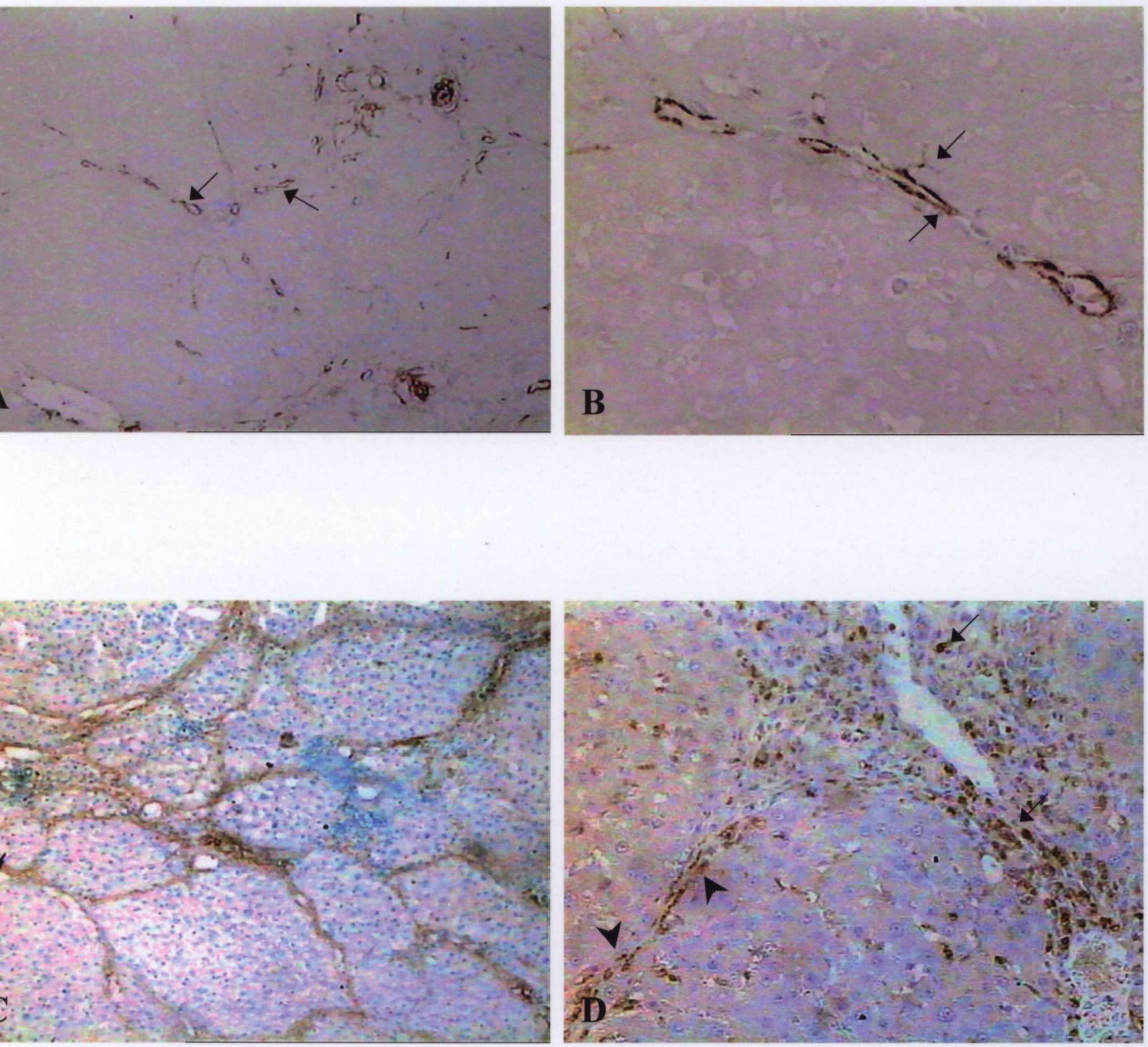

Fig. 3

Fig 4 – **A**: Type I collagen is evidenced by immunofluorescence in the fibrous septa which radiate from portal space in this example of very early *C. hepatica*-infection in a rat. 100X. **B**: Reveals a similar picture for type III collagen, 200X. **C** and **D**: the prominent presence of proliferatiung vessels is revealed by these immunofluorescence preparations with anti-type IV collagen. 100 and 200X, respectively. **E**: Laminin. **F**: Fibronectin. All pictures taken at the 25th day of infection.

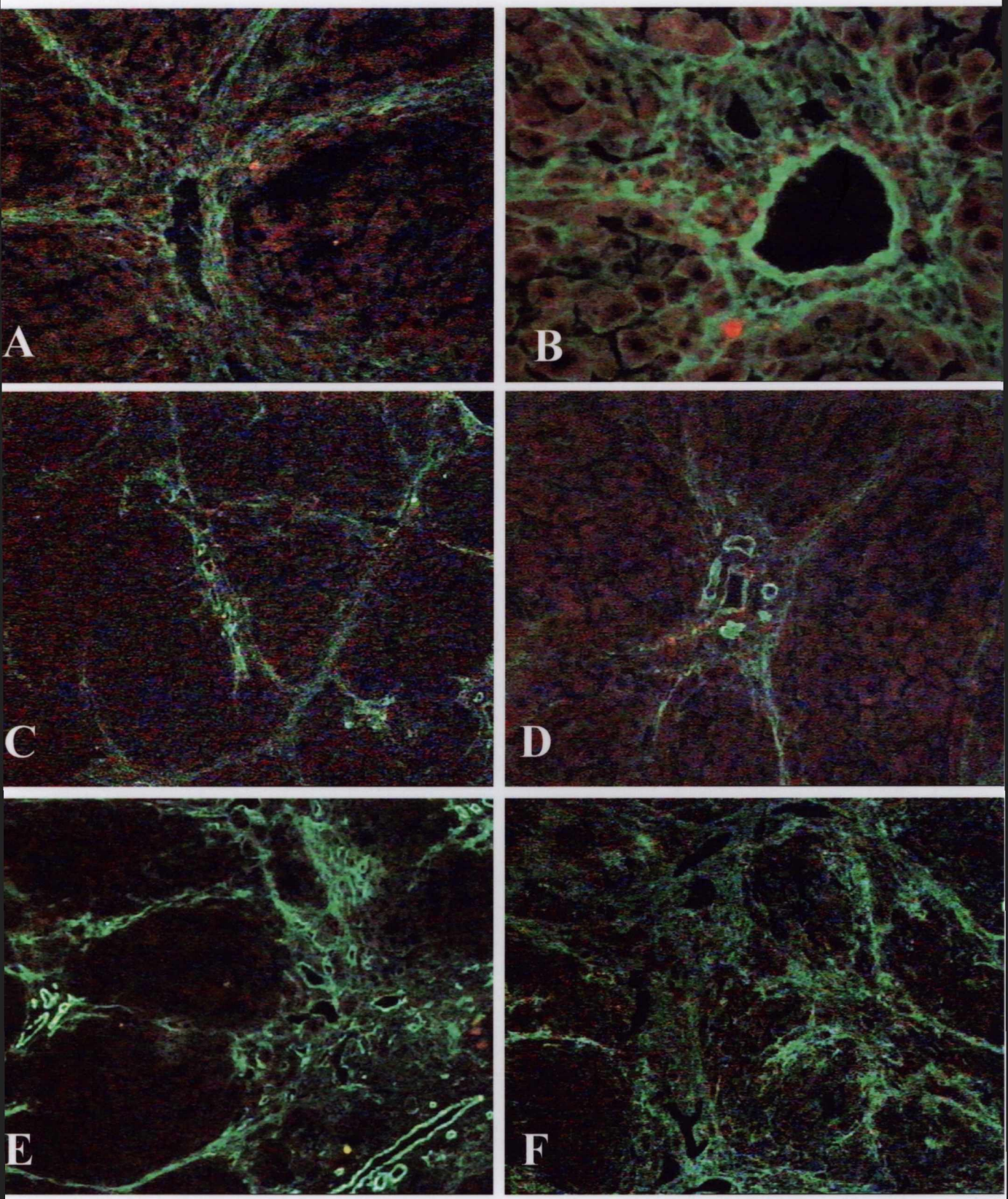

Fig. 4

Fig. 5 – **A**: The polymorph cellular composition of the early septum is illustrated in this micrograph. There are endothelials cells (EC), eosinophil (E) and an apoptotic cell (Ac).**B**: A polymorphonuclear eosinophil is showed at the center of the picture, (asterisk) while a fat-storing cell appears at lower left (arrow). **C**: Fibroblasts (asterisks) and a largely degranulated eosinophil (arrow) are present in the middle of cellular debris. **D**: A small vessel appears surrounded by enlarged, proliferating endothelial cells (asterisks); Electron microscopy, A, C and D 4,400X; B 7,000X.

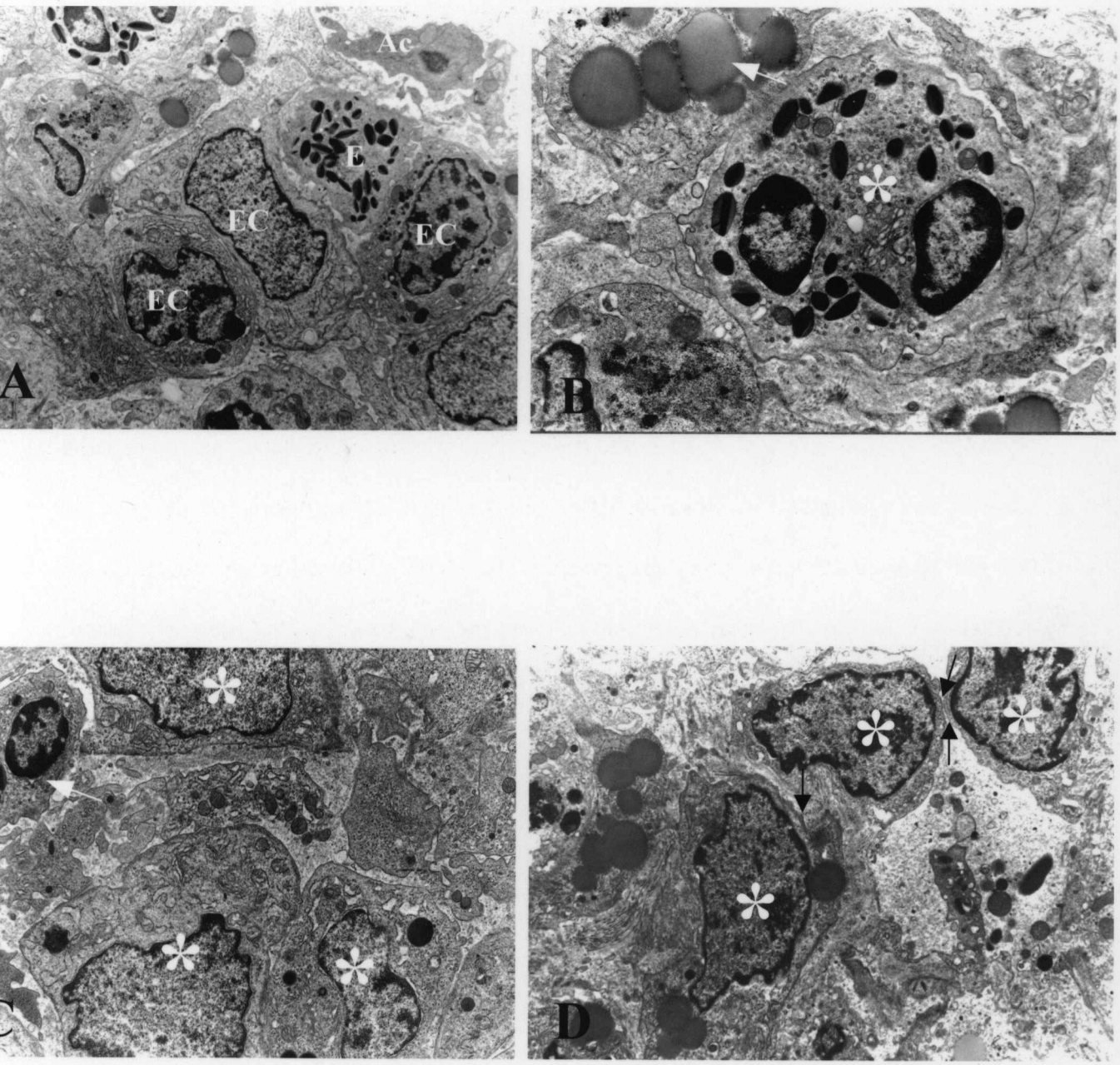

Fig. 5

4 MANUSCRITO II

Significance and fate of septal fibrosis of the liver.

(An experimental study on prolonged *Capillaria-hepatica*-infection in rats).

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Summary. *Capillaria hepatica* infection in rats induces a process of systematized septal fibrosis of the liver, that run a progressive course during the first three months of infection. From then on, its evolution has not been studied. Present investigation looks into functional and morphological changes of septal fibrosis in prolonged *C. hepatica* infection, to inquire on its fate and significance. Adults Wistar rats were sacrificed 3, 6, 9 and 12 months following inoculation of 800 embryoned eggs of *C. hepatica*. Besides routine histology, liver sections were submitted to immunohistochemical, immunofluorescence and ultrastructural techniques for the identification of cells and extracellular matrix components present in the fibrous septa. Septal blood vessels were studied by portal vein injection of India-ink. Hepatic functional profile and levels of anti- *C. hepatica* antibodies were made from collected serum. Results revealed extinction of parasitism by the 6th month, with considerable reduction in the number of cells an in the amount of collagen in the septa. From then on the picture stabilized and no further modifications were noted in subsequent months. Septal fibrosis persisted throughout the time of experimentation (12 months). Fibrosis was seen as a supporting stroma for septal vessels that conducted portal blood directly into sinusoids. In this case, persistence of fibrosis was related to its morphological and functional association with blood vessels.

INTRODUCTION

A peculiar type of septal fibrosis of the liver regularly occurs in *Capillaria hepatica*-infected rats (Ferreira & Andrade, 1993). During its life cycle, this helminth dies out inside the liver, around the 20th day of infection, soon after sexual maturity and egg laying. Necrotic-inflammatory lesions are then formed around the disintegrating parasites and their eggs. When such focal parasitic lesions start exhibiting signs of encapsulation and progressive resorption, which usually takes place about the 25th to 30th day following infection, thin and long fibrous septa begin to sprout out from portal spaces throughout the liver, dividing the hepatic parenchyma into several polyhedral portions of different sizes. It has been suggested that this process is progressive and will end in true cirrhosis (Ferreira & Andrade 1993). Early studies have noted that end-stage cirrhosis can be the outcome of severe *C. hepatica* infection in rodents (Luttermoser, 1938). Significantly, Lammler et al. (1974) pointed out that these late lesions are difficult to be explained, since at this time egg production is ceasing and acute focal lesions are no longer observed. As a matter of fact, when *C. hepatica*-induced lesions were followed through different intervals, from 1 to 3 months, the process of septal fibrosis was seen to increase progressively with time (Ferreira & Andrade, 1993). However, the outcome of *C. hepatica*-induced septal fibrosis is not known, since sufficiently prolonged studies have not been made on this regard.

Also, in a much more studied model of hepatic septal fibrosis, the one induced in rats by repeated intraperitoneal injections of whole pig-serum or its albumin fraction, only relatively short term studies have been attempted (Paronetto & Popper, 1966).

Whether septal fibrosis, as seen in both experimental models just mentioned, will continuously evolve toward cirrhosis or will eventually stop and disappear, are questions

of considerable interest to the study, not only of the respective models, but to hepatic fibrosis in general.

The present investigation followed the changes of septal fibrosis in the liver of rats infected with *C. hepatica,* at several time-intervals, from 3 months to over a year. While the amount of collagen considerably decreased with the extinction of parasitism, septal fibrosis persisted. It seemed that the liver became adapted to a new portal vasculature, compatible with a normal function. The observed data may also have a bearing on the problems concerning reversibility of cirrhosis.

MATERIALS AND METHODS

Animals - A total of 54 healthy, outbred Wistar rats, weighing 200-500 g, of both sexes, were used. They were maintained in good housing conditions, with free access to a commercial balanced diet and water. All the animals were submitted to infection with 800 mature *C. hepatica* eggs, administered by a gastric tube. These eggs were isolated from the livers of experimentally infected mice, through homogenization in saline followed by repeated washing and sedimentation. The clean isolated eggs were kept in a humidified Petri dish at 25-28^0 C. during a period of 28 days for embryonation.

Under total anesthesia and asseptic conditions, the animals were submitted to a surgical biopsy, with removal of a liver lobe at different times after inoculation, as follows: 3 months (n=15), 6 months (n=15), 9 months (n=9) and 12 months (n=15). At each time-point, blood was collected from the tail vein, for serum samples. Also, two animals were sacrificed for the study of the portal vasculature, through the injection of India ink directly into the main portal vein. The injection was made manually until the liver appeared uniformwely black. India-ink was diluted (50-50) in 15% gelatin.

Normal intact control rats were used for the obtaining of serum samples and for the studies of the portal vessels after India-ink perfusion.

Histopathology – Fragments of the liver were fixed for at least 48 hours in buffered (pH 7.2) 10% formalin. Paraffin-embedded sections were stained with hematoxylin and eosin, sirius-red for collagen, the PAS-method with and without diastase digestion, Perls' method for iron, toluidine-blue for cellular metachromatic granules and orcein for elastic fibers.

Transmission Electron Microscopy – Tiny fragments of liver tissue (about 1 mm^3) were immediately fixed by immersion into 4% glutaraldehyde in 0.2M cacodylate buffer, pH 7.4, for 1 hr at 4 °C, washed in buffer and postfixed with 1% osmium tetroxide, dehydrated in graded concentrations of acetone and embedded in Polii-bed 812 (Embedding Media Polysciences, INC) Selected ultrathin sections (50-70 nm) were made with a Reichert (Ultratome Supernova Leica) ultramicrotome and mounted on uncoated copper grids, contrasted with uranyl acetate and a lead citrate. Specimens were examined in a Zeiss EM-9 electron microscope, which was operated at an acceleration voltage of 50 kV.

Immunofluorescence - Fragments of liver tissue were immediately placed in liquid nitrogen for a few minutes and them kept frozen at -70^0 C in airtight boxes, until the moment they were sectioned in a cryostat at -20^0 C. The sections were submitted to the indirect immunofluorescence technique for the demonstration of collagen isotypes (I,III and IV), laminin and fibronectin. The specific anti-sera were polyclonal, obtained in rabbits (Institute Pasteur, France). They were used in dilution varying from 1:40 to 1:100. Details concerning their preparation and tests of specificity appeared elsewhere (Andrade et al. 1992). Secondary fluoresceinated anti rabbit-IgG was commercially obtained from SIGMA, USA.

Immunohistochemistry – For the demonstration of factor VIII, dendritic cells and ED3, fragments of the liver were immediately embedded in Tissue–teck (OCT compound-Miles Inc. Diagnostic Division, Elkhart, USA), frozen into liquid Nitrogen, and cryopreserved in a freezer at –70°C until the moment of sectioning.

Cryostat and paraffin sections of 6μm were obtained in a cryostat at –20°C were mounted on slides previously treated with Poly-L-Lisin at 10% (Sigma St. Louis, Mo. USA), fixed in dehydrated acetone, treated with PBS containing 0.1% Saponin (Sigma St. Louis, Mo. USA) and 1% Bovin Serum Albumin (BSA) (Sigma St. Louis, Mo. USA). For blockade of non-specific ligations sections were treated with BSA - 30% in PBS for 20 minutes at room temperature.

For the demonstration of SM-α actin, TGF-β, TGF-β-R, desmin, vimentin and PDGF, paraffin sections of formalin-fixed tissue were used. Antigen retrieval was accomplished through micro-wave treatment in citrate buffer at pH 6.0.

Sections were incubated with the primary antibodies (see Table 1) overnight, at 4°C in an humidified chamber. Primary antibodies were diluted in 2% BSA in PBS (pH 7.4). After washing into PBS and no fat milk , during 20 minutes for a blockade of non-specific ligations. The slides were then incubated with the Kit Peroxidase conjugate to anti-mouse and anti-rabbit immunoglobulins (Dako envision system – labelled polymer (K1392). Blockade of the endogenous peroxidase was done with 0.3% H_2O_2 for 30 minutes, at room temperature. The color was developed with 0.06% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, Mo-USA) and 0.06% H_2O_2 plus 1% dimethylsulphoxide (Sigma, St. Louis, Mo-USA). Sections were counterstained with 1% methyl-green for 2 minutes dehydrated and mounted with Permount. Control sections in which primary antibody was either omitted or replaced by normal rat serum, were used.

Functional Liver Tests – The serum concentrations of transaminases (ALT and AST), alkaline phosphatase (ALK P), and gamma-glutamyl transpeptidase (γ-GT) were determined in a Hitachi 917 automatic analyzer.

Hidroxyprolin determination - Biochemical determination of hydroxyproline content was made according to the colorimetric method of Bergman & Loxley (1963).

Serum antibodies – The levels of serum antibodies against *C. hepatica* were determined by un enzyme-linked immunosorbant assay (ELISA) to test for detection of total Ig antibodies, by using a goat anti-rat IgG conjugated to peroxidase (Sigma). The plates were sensitized overnight with 10μg/ml/per well of *C. hepatica*-egg antigen, diluted in carbonate buffer, pH 9.9. Lecture was made on a microplate reader "Molecular devices-Thermomax" spectrophotometer, under wave-length 450nm, connected to a computer with MDS-Soft Max (version 3.0).

Statistical analyses. Data were analyzed using Student's *t* test, for normally-distributed data, or the Mann Whitney test, for non-normally distributed data, as indicated in the text. Differences were considered significant when $p<0,05$.

RESULTS

Multifocal parasitic lesions, containing worm debris and immature eggs were already well-encapsulated by 3 months after inoculation. They almost disappeared at 6 months. Only clusters of eggs packed inside scattered, dense, small fibrous scars, with or without a few calcified foci, remained by the 6th months on. Three months after infection, septal fibrosis appeared throughout the liver. It was represented by thin and long fibrous septa crisscrossing the hepatic parenquima. These thin septa connected portal spaces to portal spaces, portal spaces to central veins and central veins to central veins. Some septa

appeared projected from the capsule of old parasitic scars, and from the external capsule of the liver. The portions of hepatic tissue circumscribed by septal fibrosis, maintained the one-cell thick normal pattern of the liver parenquima. Six months after infection, the microscopic appearance of septal fibrosis had changed. In H & E preparations fibrous septa were hardly discernible, since the place occupied by fibrosis appeared as a thin cleft, with a few fusiform cells here and there. Sirius-red stained slides easily disclosed that septal fibrosis was still present as delicate, thin treads of red-stained fibers. PAS-diastase treated slides disclosed fine blood vessels cursing along the fibrous septa (Fig. 1A). The septa had a characteristic wavy and interrupted distribution. The presence of elastic fibers within these septa was observed in orcein stained preparations (Fig. 3C). These overall aspects tended to persist when materials taken from 9 and 12 month-old infections were examined. In short, besides the diminution in the amount of septal collagen from the 3rd to the 6th month following infection, all the other parameters considered, tended to remain the same, 9 and 12 months after infection. Isolation and cultivation of eggs taken from 3rd month-old infection yielded embryonation, which did not occur when eggs from 6, 9 or 12 month-old infections were tested. Determination of hidroxyprolin confirmed the impression from histological examination, showing a statistically significant difference in collagen concentration as compared to specimens taken from more prolonged infections, however from the 6th month on the hydroxyprolin measurements were about the same, indicating an stabilization of the process of collagen degradation (Fig.6).

Collagen isotypes I and III were present within the fibrous septa at all observational stages. Collagen type IV and laminin marked basement membranes and thus disclosed the rich vascularization of the fibrous septa and also their permanence throughout the time of

observation. Fibronectin in the fibrous septa presented decreased concentrations along the time of examination, as a matter of fact in a way comparable to what happened to the other components of the extracellular matrix evidenced by immunofluorescence.

The cellular elements in the fibrous septa were scarce even at the 3rd month of infection, and this was even more evident during the subsequent periods. No special aspects concerning the number and distribution of the cellular components of the septa, as identified by imonohistochemistry, were apparent. Cells positive for desmin, vimentin, PDGF, TGF-β, TGF-β-R, ED3, and dendritic cells (Fig 1 C and D) were few and appeared occasionally within the thin fibrous septa. Actin was positive for a few cells in the septa, but appeared prominent revealing the muscular walls of blood vessels (Fig. 3D), the endothelial cells of which appeared positive for factor VIII (Fig.1B). Late septa, fom the 6th to the 12th months, practically contained collagen and blood vessels (Fig. 3A, B).

Ultrastructurally, the septa from the 3rd month exhibited a mixture of several cell types, with predominmance of fibroblasts and a few myofibroblasts (Fig.4A) Other cells eventually identified in the septa included fat-storing cells (HSC), mast cells, eosinophils and lymphocytes. From the 6th month on, the septa contained densely packed collagen fibers and one or other of the cells previously mentioned (Fig. 4B).

Sections of the liver previously injected with India-ink revealed that the fibrous septa contained vessels that terminated directly into sinusoids. Some of these vessels appeared straight, thin, without collaterals, running for some distance within the septum, before dividing into several terminal ramifications towards the sinusoids. These aspects were observed in all specimens taken from infected rats (Fig.5). Normal controls showed that portal vessels tended to form and arcing structure near the parenchyma limiting membrane before giving off short communications to the sinusoids.

The exploration of liver function failed to reveal major alterations. The behavior of serum anti-C. hepatica antibodies is depicted in (Fig. 7).

DISCUSSION

Shibayama & Nakata (1992) demonstrated that hepatic fibrosis induced by pig-serum injections into rats did not have a functional expression. By measuring oxygen consumption, the velocity of blood perfusion, and other parameters, they concluded that fibrosis by itself does not disturb liver function. However, it is important to test the evolutionary potential of a fibrosis. Pig serum fibrosis in rats may vary in intensity and sometimes mimics a histologic picture of cirrhosis (Paronetto & Popper 1966, Andrade 1991). The preliminary data obtained in the present study also did not reveal disturbance of liver function in septal fibrosis induced by *C. hepatica* infection in rats. The significance of fibrosis in these mentioned models is incompletely known. It is important to determine its fate, especially regarding basic mechanisms of fibrosis production.

The persistence of *C. hepatica*-induced septal fibrosis in the liver, during a prolonged time after the parasitic infection had became extinguished, is intriguing. Several studies have demonstrated that hepatic fibrosis can be reversible (Abdel-Aziz et al. 1990, Andrade, 1994,Ozaki et al. 2000, 2001). Studies on experimental hepatic schistosomiasis have demonstrated that the liver can be almost completely cleared from fibrosis within six months after curative treatment (Andrade & Grimaud 1986). It takes longer in cases of advanced human schistosomiasis, but even severe periportal (pipestem) fibrosis of hepatosplenic schistosomiasis can be reversed (Andrade 1992). However, the debate is still lingering regarding human and experimental cirrhosis. Although Wanless et al. (2000) listed a series of histological findings, collectively called the hepatic repair complex,

supposed to represent parameters of cirrhosis in regression, no one has ever documented the transition of a cirrhotic liver to a normal one.

When the present study was made 6 months after *C. hepatica* infection, the data showing extinction of parasitism and decrease of septal fibrosis, left the. impression that the whole structure of the liver would subsequently return to normal. However, septal fibrosis became stabilized for the next 6 months. Fibrosis then appeared to be merely a supportive stroma of a new vascular disposition. Probably aggression induced evolutionary forces of adaptation and the liver of a rat became reminiscent of that of other species, like the pig, camel, etc.

Speculations apart, it can be concluded that associated vascular changes may be a decisive factor regarding reversibility of fibrosis. After removal of its cause, excess fibrous tissue tends to be removed, but that part supporting vessels that are physiologically viable would be preserved. Therefore, cirrhosis is clinically reversible, as recently discussed by Friedman (1999), although still morphologically irreversible.

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Table 1: Antibodies and their sources used in this study

Antibody	Dilution	Source
Mouse anti-TGFR1-β	1:20	NOVO CASTRA
Mouse anti- smooth muscle actin (clone 1A4)	1:100	DAKO
Anti-vimentin (anti-human - clone VIM 3B4)	1:25	DAKO
Mouse anti-TGF beta (anti-human – clone TGFB17)	1:80	NOVO CASTRA
Rabbit anti- PDGF (policlonal, clone AB-1)	1:10	CALBIOCHEM
Anti- desmin (Clone D33)	1:10	DAKO
Mouse anti-macrophages (anti-rat clone ED3)	1:80	BIOSOURCE INTERNATIONAL
Anti- Fator VIII (mouse anti-human)	1:100	DAKOPATTS
Mouse McAb anti- dendritic cells (clone OX-6)	1:80	SERA-LAB

Fig 1 – **A**: The vascular nature of the thin fibrous septum found in the liver of rat with prolonged (9 months) *C. hepatica* infection is evident in this picture. PAS diastasis, 100X. **B**: Presence of cells expressing immunohistochemical reactivity for factor VIII (arrows) within fibrous septa, one year after *C. hepatica* infection.100X. **C**: and **D**: the same for dendritic cells (arrows). C 100X, D 200X.

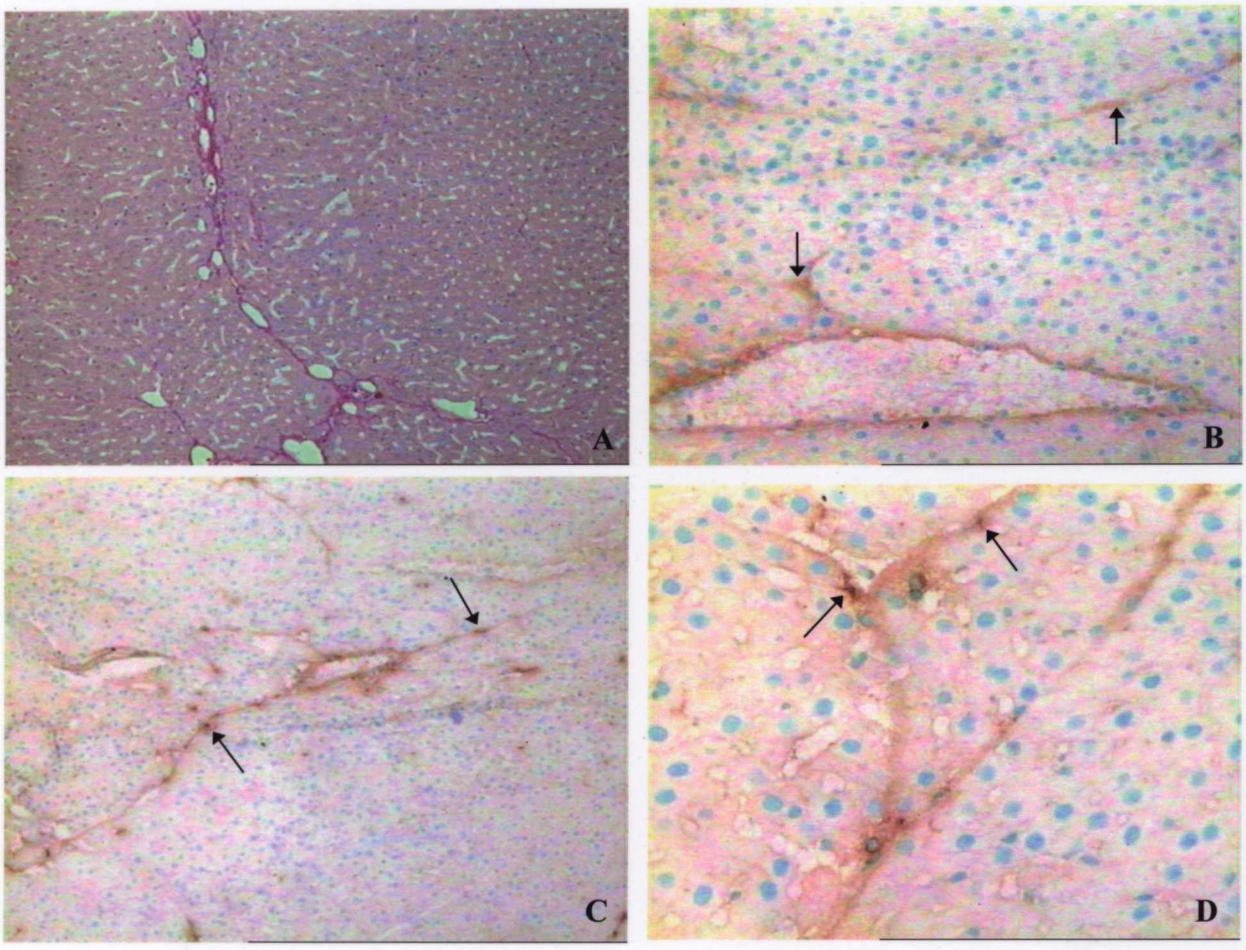

Fig. 1

Fig 2 – It shows the progressive decrease of the expression of desmin snd TGF-β in hepatic septal fibrosis induced by *C. hepatica* during prolonged infection of the rat. **A** and **B** represent the presence of desmin after 6 months and one year of infection. **C** and and **D** represent the same for TGF-β. All pictures taken at magnification 100X.

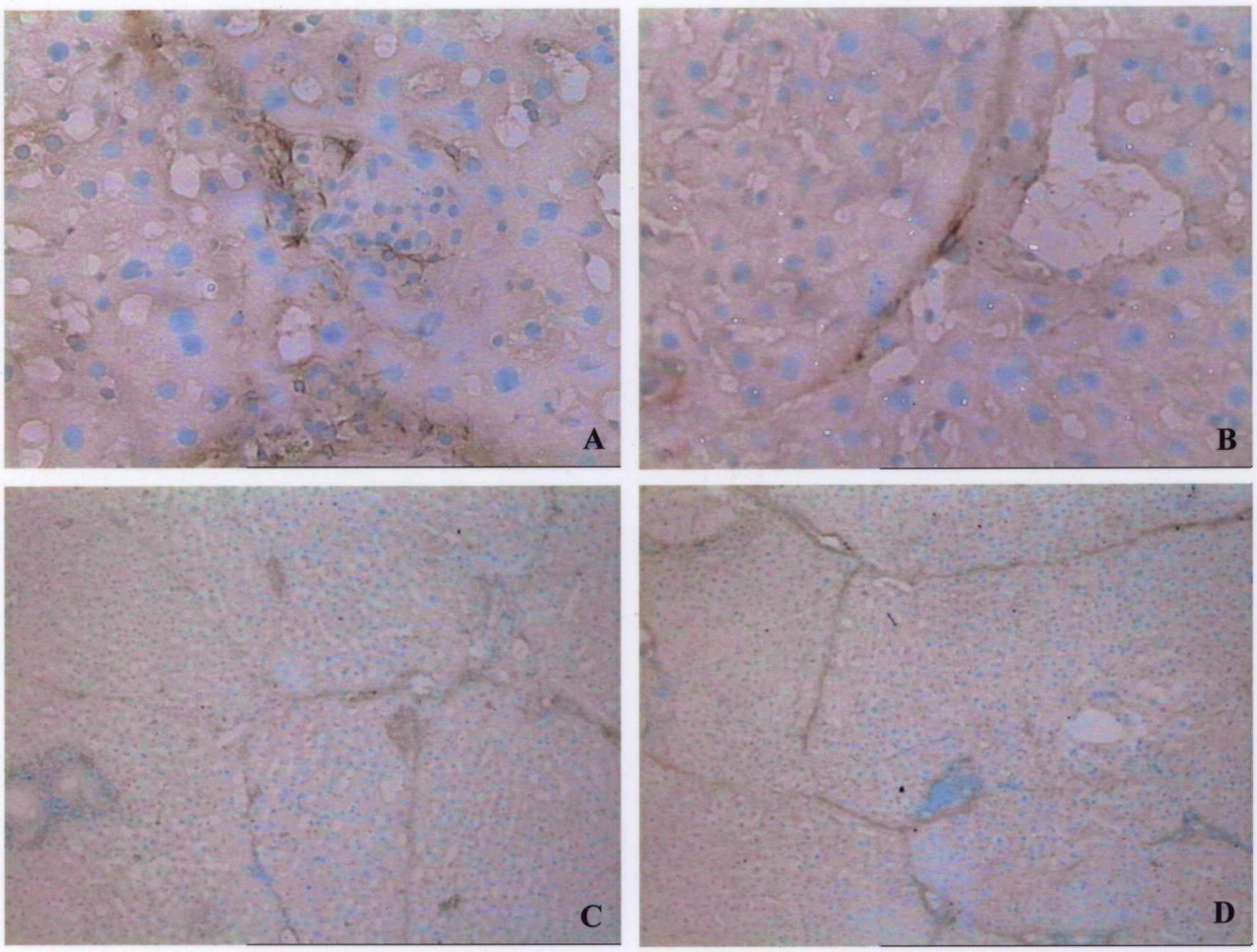

Fig. 2

Fig 3 – The continuous presence of blood vessels within thin fibrous septa persisting in the livers of rats infected with *C. hepatica* is demonstrated by different morphological methods. **A**: vascular basement membrane immunofluorescent staining for laminin reveals large vessels in portal space and small ones forming a curved row within a septum, 200X. **B**: fibronecting in extracellular matriz helps delineating several small holes (blood vessels) in the fibrous septa. 200X. **C**: Blood vessels appear dark stained for elastic fibers in portal space and within the septa (arrows), which also contain some elastic fibrils. Orcein stain. 100X. **D**: Immunohistochemical staining for α-actin discloses the thin persistent septa. (arrows) and a few portal blood vessels (arrow heads). 100X. All examples taken from 9 month-old infections.

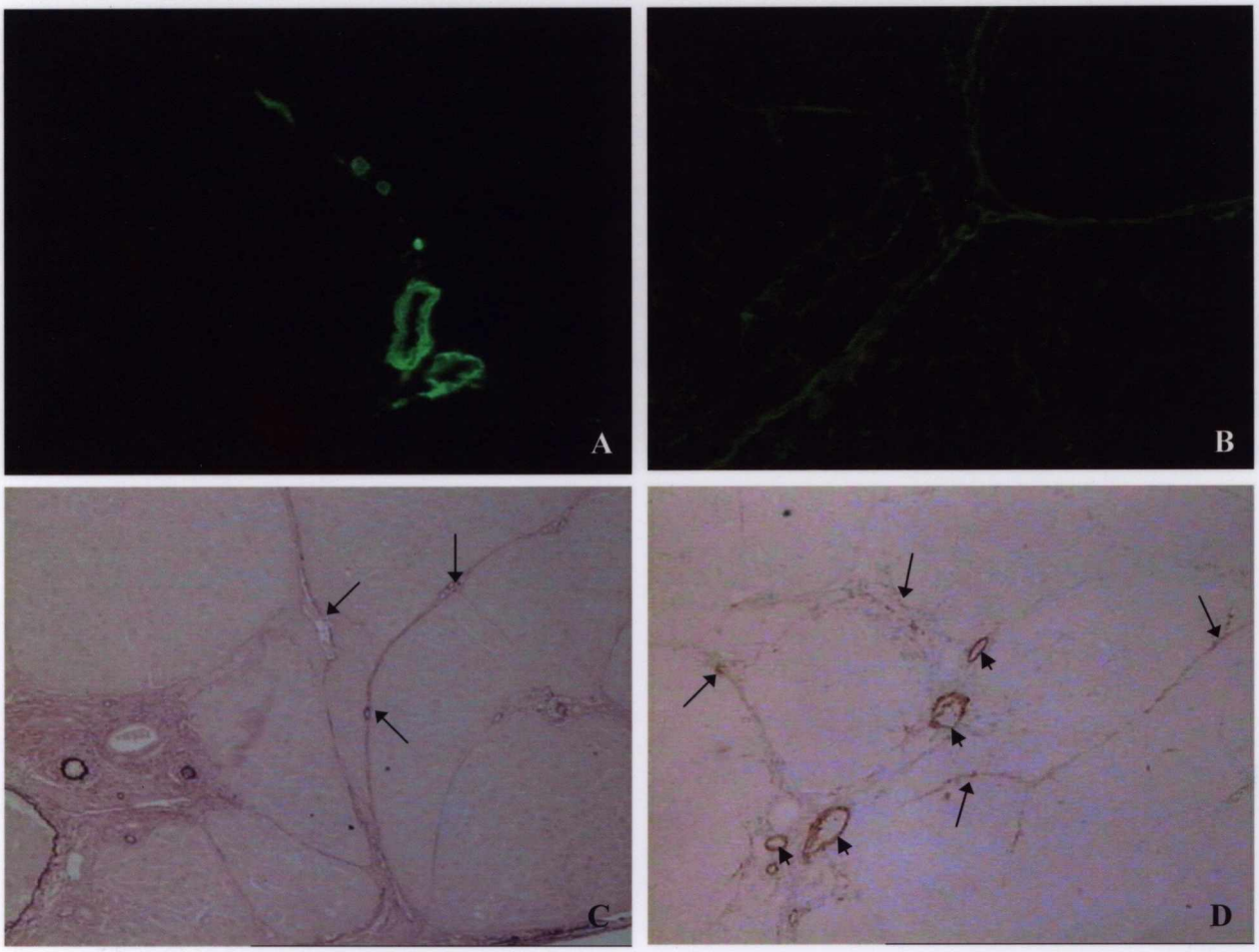

Fig. 3

Fig 4 – Electron micrographs. **A**: a cellular septum from a 3 month-old infection reveals parallel-disposed fibroblasts (arrows) and loosely packed collagen stroma (asterisks). 3,000X. **B** – By the 6 month of *C. hepatica* infection a representative septum presents as an acellular packing of parallel collagen fibers (arrows). There are collagen fibers accumulated in the Disse space with fibers which crossed at a different orientation (asterisks). 4,400X. **C**: A collagenous septa, viewed at 9 months after infection, running in between normal-looking hepatocytes 7,000. M = mitochondrium, ER = endoplasmic reticulum.

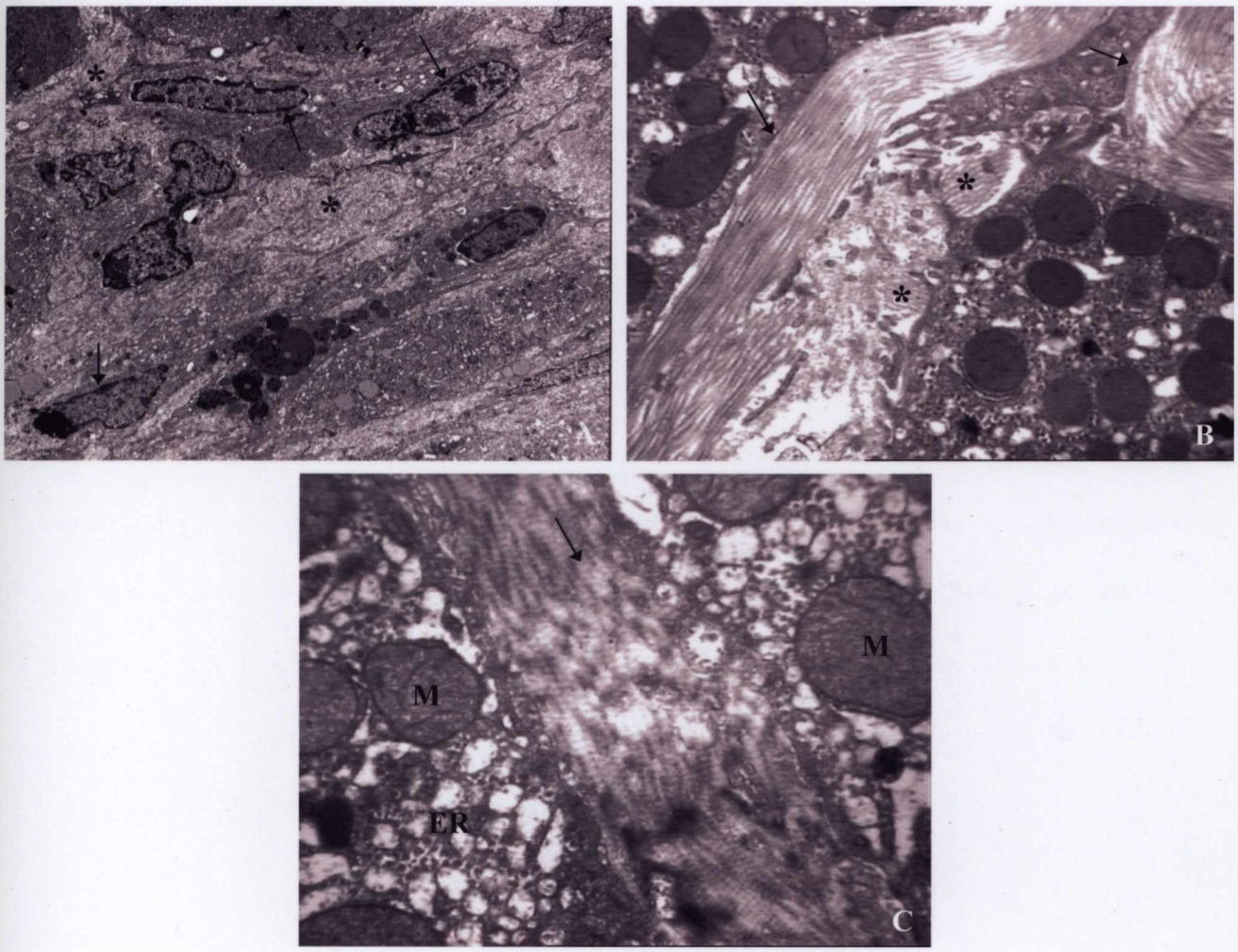

Fig. 4

Fig 5 – Hepatic vessels appear marked by Indian-ink which has been injected into the main portal trunk. The pictures illustrate the filling of septal vessels and sinusoids, indicating that they are easily perfused from circulating "blood" within the septa, even when nodular hepatocellular structures are present and the septa form a curved capsule around them (arrows)..Old C. hepatica infections (9-12 monts). Counterstained with H & E. **A** and **D** = 100X; **B** and **C** = 200X.

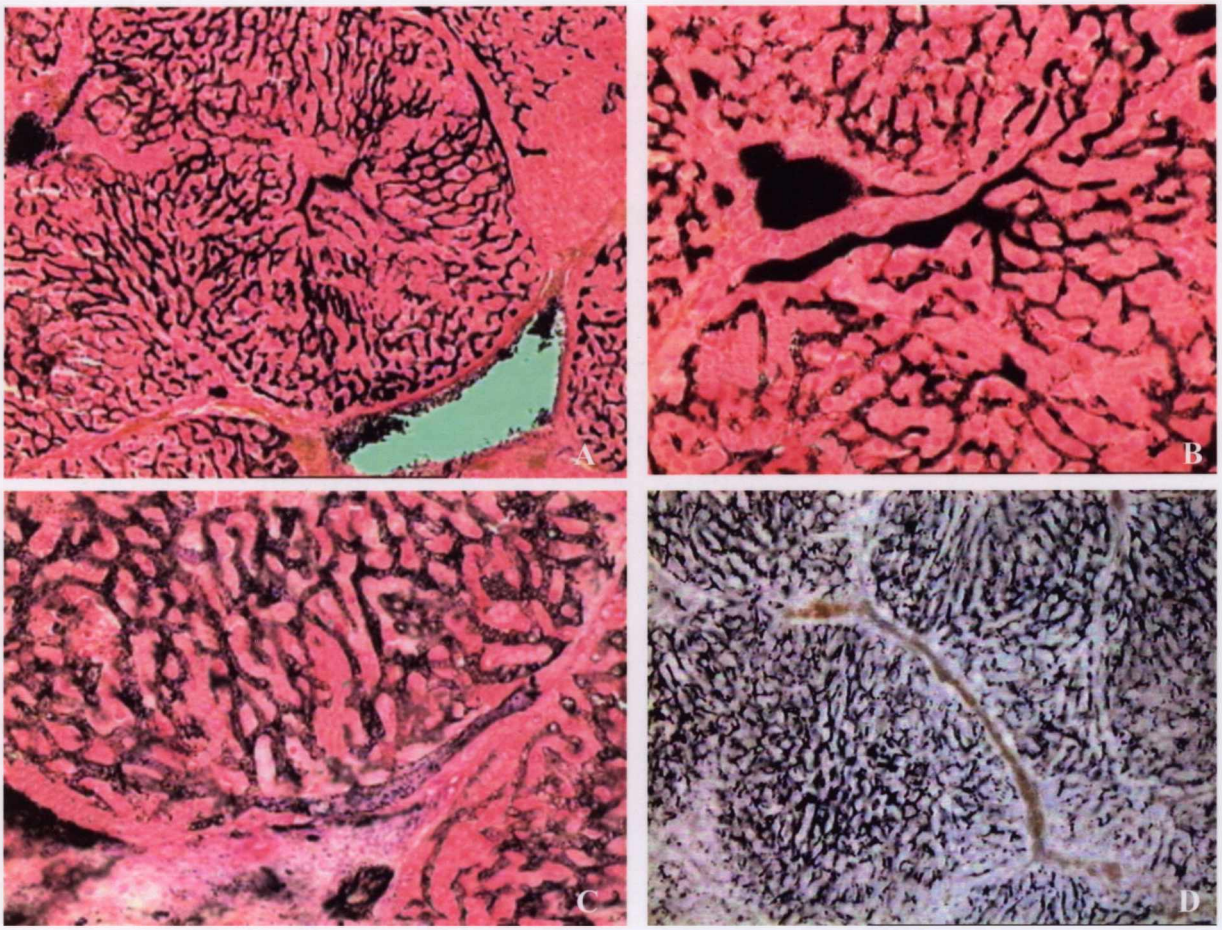

Fig. 5

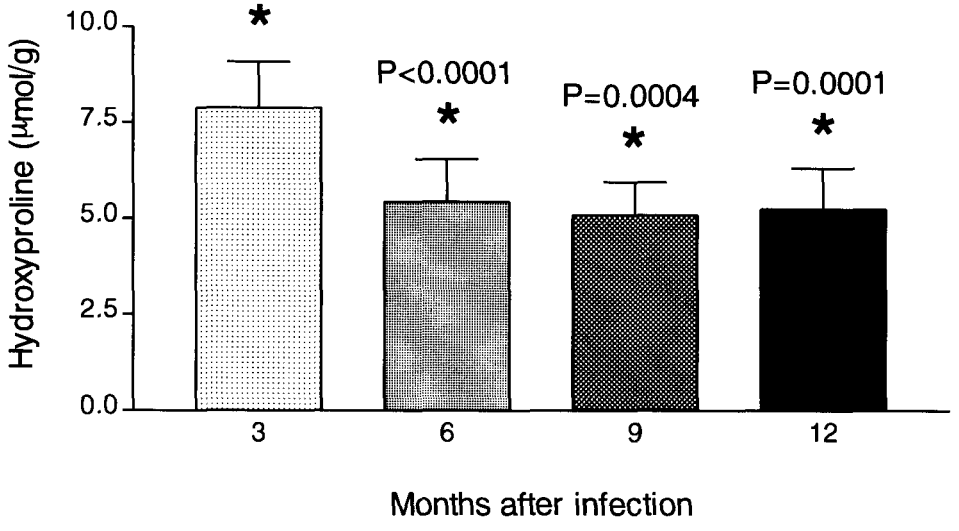

Figure 6 : Measurement of liver collagen concentration by hydroxyproline determination. Liver fragments of Wistar rats were obtained 3, 6, 9 and 12 months after infection. The hydroxyproline content per gram of liver from individual rats were determined by colorimetric assay (Method of Bergman & Loxley, 1963)

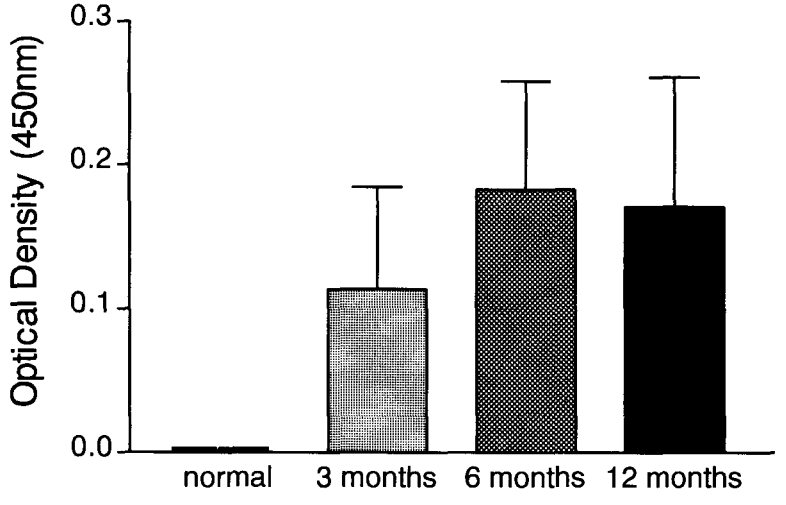

Figure 7 : Levels of antibodies detected in the serum of the rats after 3, 6 and 12 months of the infection

5 DISCUSSÃO

Acompanhando dia-a-dia a infecção por *C. hepatica*, a partir do 13º dia pós-infecção, foi possível surpreender os primeiros sinais de formação da fibrose septal, os quais surgiram no 25º dia (Manuscrito I). Sendo assim, o estudo das fases mais iniciais do processo de fibrose septal foi concentrado numa faixa de quinze dias, que compreendeu desde o 25º até o 39º dia pós-infecção. Dessa forma, através de biópsias realizadas a cada dois dias; inclusive com re-biópsias após sete dias, o processo foi acompanhado, servindo cada animal como seu próprio controle da evolução inicial da fibrose.

Na tentativa de melhor compreender o significado e o potencial evolutivo da fibrose no modelo *C. hepatica*-rato, os estudos continuaram com um segundo experimento (Manuscrito II) onde foram investigadas fases mais avançadas da infecção, com exames realizados após 3, 6, 9 e 12 meses pós-inoculação dos animais.

Como resultado geral, várias noções que vínhamos acumulando após cerca de 5 anos de estudos com o modelo, tiveram que ser corrigidas. A primeira delas diz respeito ao papel relativo que as células estreladas poderiam desempenhar na patogenia da fibrose septal. Por estarem os septos fibrosos situados entre traves hepáticas, onde se situam os sinusóides com suas células estreladas, bem como pelo papel preponderante que as ditas células desempenham na fibrogênese, seria natural supor que elas estariam envolvidas na primeira linha do processo fibrosante. A princípio a possibilidade de uma estimulação do eixo células de Kupffer/células estreladas de Ito por produtos liberados das lesões parasitárias focais,

aparecia como uma hipótese das mais prováveis (FERREIRA e ANDRADE, 1993). Ficava todavia difícil se admitir que tal estímulo não fosse difuso, mas que afetasse seletivamente alguns sinusóides, aparentemente aqueles situados na zona III do ácino hepático. Foi levantada então a possibilidade de que a baixa concentração de oxigênio existente na zona III facilitasse a acumulação do colágeno sintetizado pelas células estreladas estimuladas, enquanto a sua degradação seria rapidamente feita nos demais setores. A situação das células de Kupffer também não facilitava a hipótese do eixo Kupffer/células estreladas. Estas células não mostraram sinais de estimulação e proliferação, nem de concentração nas proximidades dos septos, quando visualidades após marcação com tinta da China ou imunohistoquimicamente com ED3.

BHUNCHET e colaboradores, (1996), investigando se a fibrose hepática induzida por soro de porco em ratos era causada por uma reação imune ao soro de porco demonstraram, através de marcação para ED1 (anticorpo específico para macrófagos/monócitos), que havia numerosos macrófagos e monócitos dentro do septo recém-formado e das partes fibróticas da cápsula hepática e poucos nas partes não fibróticas da mesma. Nossos resultados com o modelo de fibrose septal induzida pela C. *hepatica* são diferentes dos relados por BHUNCHET e colaboradores, (1996), na fibrose induzida por injeções intraperitoneais de soro de porco, pois, foi demonstrado, tanto imunohistoquimicamente através de marcação para ED3, como através de injeção de tinta da China a 10% na veia da cauda de ratos infectados com *C. hepatica* (substância utilizada como marcador do processo de fagocitose) que a distribuição dos macrófagos hepáticos se deu uniformemente por todo

parênquima, semelhante ao que aconteceu em controles intactos, e não, especificamente, na região dos septos. Estes dados não excluem que haja um estímulo, mas não são de cunho a apoiar esta hipótese.

A presente investigação das fases mais iniciais da formação dos septos veio revelar também que, embora os mesmos possam se originar das cápsulas das lesões parasitárias focais, da cápsula externa do fígado e mesmo das veias centrais, o local preferencial é a zona periportal. Daí partem os septos, irradiando-se para o interior do parênquima. Há uma proliferação de células mesenquimais nas áreas periportals. Estas células se enfileiram e formam os septos iniciais que então aparecem celulosos e com apenas algumas raras fibrilas colágenas. Observam-se nesta fase uma grande variedade de tipos celulares, com predominância de fibroblastos e células endoteliais, mas onde as células estreladas são raras, após exames histológicos, imuno-histoquímicos e ultraestruturais. Mas, o elemento que se destaca, especialmente quando se aplicam técnicas que evidenciam componentes vasculares, são os capilares, vênulas e arteríolas.

A angiogênese, ou seja, a formação de novos vasos sangüíneos, é um processo complexo que envolve a proliferação e migração de células endoteliais. Este fenômeno é requerido durante a cicatrização de tecidos lesados, como também, durante a proliferação de células cancerosas, para suprimento de nutrientes e oxigênio (ALGIRE et al., 1945; FOLKMAN, 1995 a, b). A proliferação de células endoteliais é predominantemente regulada pelo fator de crescimento do endotélio vascular (VEGF) (YAMANE et al., 1994), embora outros fatores angiogênicos tais como fator de crescimento derivado de plaqueta (PDGF) e o fator de crescimento do fibroblasto (FGF) também

participem nesse processo (FOLKMAN & KLAGSBRUN, 1987; FOLKMAN 1995 a, b; GOSPODAROWICZ et al., 1987; LEVEEN et al., 1994; SHIBUYA, 1995). Segundo TANIGUCHI e colaboradores, (2001), o VEGF promove a proliferação de hepatócitos, após hepatectomia parcial, através da reconstrução dos sinusóides hepáticos pela proliferação das células endoteliais sinusoidals.

Em seus estudos pioneiros, RAPPAPORT e colaboradores (1983), mostraram que o desenvolvimento de cicatrizes no fígado cirrótico era invariavelmente acompanhado de uma intensa proliferação vascular. Suas observações têm sido plenamente confirmadas por outros autores mostrando que nos tecidos cirróticos, os nódulos regenerativos são envolvidos por um denso plexo vascular (HARATAKE et al., 1991; YAMOTO et al., 1984).

Tem sido demonstrado que a hipóxia é o principal indutor de expressão de VEGF que por sua vez, estimula a proliferação de capilares para aumentar a distribuição de oxigênio (SHWEIKI et al., 1992). *In vitro*, a expressão de VEGF aumenta especificamente em células expostas a hipóxia (BROGI et al., 1994; SHWEIKI et al., 1992). *In vivo*, a expressão aumentada de VEGF ocorre em uma variedade de processos patológicos caracterizados por neovascularização relacionada a hipóxia. Por exemplo, a produção de VEGF é aumentada durante a cicatrização (BROWN et al., 1992), devido à ocorrência de baixa tensão de oxigênio na parte central de um ferimento após ruptura da vasculatura local (KNIGHTON et al., 1983). Segundo BROGI e colaboradores (1995), mecanismos parácrinos iniciados pela hipóxia podem operar para induzir a angiogênese em tecidos isquêmicos. Acrescentam ainda, que estes mecanismos parácrinos podem operar *in vivo* não apenas

em áreas hipóxicas de tumores, mas, também, em condições não-neoplásicas, tais como na doença vascular periférica isquêmica.

ROSMORDUC e colaboradores, (1996, 1999), e EL-ASSAL e colaboradores, (1998), têm recentemente observado uma regulação positiva do VEGF nos fígados cirróticos de pacientes com ou sem carcinoma hépato-celular sugerindo que este fator pode ser responsável pela angiogênese associada à cirrose. ROSMORDUC e colaboradores (1999), estudando o papel da hipóxia hépato-celular na indução do VEGF na cirrose biliar experimental, sugeriram que a expressão de VEGF induzida pela hipóxia de hepatócitos pode ser o evento promotor do desenvolvimento da proliferação microvascular associada com a fibrogênese hepática e pode, além disso, contribuir para o remodelamento da arquitetura hepática. Contudo, não se pode excluir um papel adicional de fatores produzidos pela ativação de células inflamatórias (NAPOLI et al., 1997; SAPERSTEIN et al.,1994), algumas citocinas e fatores de crescimento (EGF, HGF, PDGF, TGF-β) que são hábeis em estimular a expressão de VEGF em tipos celulares específicos (DOLLECKI & CONNDLY, 1991; PERTOVAARA et al., 1994). É bem conhecido que a angiogênese induzida pela hipóxia participa em muitas condições patológicas incluindo isquemia cerebral e do miocárdio, retinopatia proliferativa e crescimento de tumores (FOLKMAN, 1995). Em adição, evidências indicam que a hipóxia e a angiogênese induzida por VEGF também podem contribuir para a cicatrização e tecido de reparo (NISSEN et al., 1998; SCHEID et al., 2000). Durante a fibrogênese hepática, as células estreladas hepáticas (CEHs) são ativadas, proliferam, contraem, induzem a degradação da matriz e sintetizam colágeno (FRIEDMAN, 1999, 2000).

Recentemente, tem sido mostrado que as CEHs podem expressar VEGF e receptores para VEGF no fígado após intoxicação com CCl_4 e podem em resposta a hipóxia *in vitro* expressar VEGF (ANKOMA-SEY et al., 2000; ISHIKAWA et al., 1999). CORPECHOT e colaboradores (2002), mostraram que a hipóxia, por ela mesma, aumenta a expressão de VEGF e de colágeno tipo I em células estreladas sugerindo que a hipóxia pode estar envolvida na progressão das doenças hepáticas crônicas através da participação tanto na angiogênese, quanto na fibrogênese. Eles demonstraram ainda, que o VEGF também pode agir em células estreladas, tanto por via autócrina, quanto por via parácrina. ISHIKAWA e colaboradores (1999), já haviam demonstrado que a expressão de VEGF é aumentada em células de Kupffer e células estreladas de rato após intoxicação por CCl_4. ANKOMA-SEY e colaboradores (1998), mostraram que as células estreladas expressam receptores de VEGF durante o processo de cicatrização hepática após lesão aguda. Recentemente, muito interesse tem sido focalizado no papel das células estreladas ativadas no desenvolvimento da fibrose hepática, particularmente a modulação fenotípica para células semelhantes a miofibroblastos que expressam a isoforma α-actina de músculo liso (FRENCH et al., 1988; GEERTS et al., 1989; GEERTS et al., 1991; HINES et al., 1993; KENT et al., 1976; MAK et al., 1984; MAK & LIEBER, 1988; NOUCHI et al., 1991; RAMADORI et al., 1990; ROCKEY et al., 1992; SCHMITT-GRÄFF et al., 1991; SKALLI et al., 1989; TANAKA et al., 1991). Contudo, outras células mesenquimais que não as células estreladas parecem participar na formação excessiva do tecido conjuntivo em vários modelos de fibrose. Os fibroblastos intersticiais, incluindo as chamadas células da segunda camada encontradas

nas proximidades imediatas das paredes das veias centrais e na cápsula do fígado, e fibroblastos do trato portal, tem sido identificados como potencialmente fibrogênicos, como indicado por estudos da fibrose hepática induzida em animais por soro de porco e ligadura do ducto biliar (ABDEL-AZIZ et al., 1991; BHUNCHET & WAKE, 1992; MIYAZAKI et al., 1993).

No presente trabalho, foi verificado uma abundante celularidade dos septos em torno do 25^0 dia pós-infecção ao se examinar secções coradas com H & E. (Manuscrito I). Esse acúmulo celular era composto, predominantemente, de mononucleares diversos, com uma pequena quantidade de eosinófilos. As mesmas secções, coradas com o sírius-vermelho, demonstraram escassez de fibras colágenas, nos septos, durante este período. A importância deste fato pareceu-nos estar relacionada à possibilidade de na zona III dos lóbulos hepáticos surgirem estímulos quimiotáticos que orientam o endotélio proliferado para migrar nessa direção. Pois, as células se distribuíam de forma regular e organizada, traçando verdadeiras vias que partiam de espaços-porta em direção a outros espaços-porta e também, de uma veia central em direção a outra, por todo o parênquima, sempre acompanhadas de abundantes vasos sangüíneos (capilares, arteríolas e vênulas). O fato da aparente intencionalidade na formação dos septos foi corroborado pela ausência de estimulação dos sinusóides próximos (como citado anteriormente), o que nos conduziu a imaginar que realmente poderia estar existindo uma orientação por parte do organismo na tentativa de manter o equilíbrio microcirculatório já que, os septos só se formaram nessa região (zona III) e não em outras. Outros tipos celulares presentes, observados através da microscopia eletrônica, como as

células de Ito e os miofibroblastos, reconhecidamente participantes do processo de fibrogênese, não predominaram nos septos em relação a nenhum outro tipo celular o que corrobora com os achados de ABDEL-AZIZ et al., (1991), BHUNCHET & WAKE, (1992), MIYAZAKI et al., (1993) e TUCHWEBER et al., (1996). Apesar da escassa expressão nas fases iniciais, demonstramos a presença de algumas dessas células com marcação positiva para desmina e α-actina de músculo liso. Contrariamente, os fibroblastos e as células endoteliais (positivos para vimentina e para o fator de Von Willebrand (fator VIII) respectivamente), encontravam-se em maior número nos septos, sugerindo uma participação mais efetiva dessas células no processo de fibrogênese.

Os resultados demonstraram ainda, através de marcações imunohistoquímicas contra fator de Von Willebrand (fator VIII), α-actina de músculo liso, colágeno tipo IV e laminina (Manuscrito I), que a angiogênese parece exercer uma participação importante no processo de fibrogênese hepática no modelo em consideração. Provavelmente, fatores de crescimento como o PDGF, o TGF-β1 e o TGF-β1-R, que foram demonstradas no material de estudo, através de marcação imunohistoquímica, e que já foram descritas na literatura como participantes do processo de angiogênese e fibrogênese (DOLLECKI & CONNDLY, 1991; FOLKMAN & KLAGSBRUN, 1987; FOLKMAN 1995 a, b; GOSPODAROWICZ et al., 1987; LEVEEN et al., 1994; PERTOVAARA et al., 1994; SHIBUYA, 1995), podem estar participando em conjunto com o VEGF, como estimuladores tanto de células endoteliais, quanto de células estreladas, induzindo-as a se transdiferenclarem.

As células dendríticas intersticiais foram identificadas em órgãos não linfóides e tornou-se claro que elas são as células responsáveis por estimular as fracas respostas de células T notadas no fígado, coração e rim. As células dendríticas são pobremente fagocíticas e claramente não funcionam como macrófagos teciduais convencionais *in vivo* (HART & McKENZIE, 1990).

Nas fases recentes e mais tardias da infecção, notamos a presença de células dendríticas. Nas fases iniciais, elas foram observadas espalhadas por todo parênquima enquanto que, nas fases mais tardias eles diminuíram em número e se concentraram mais nos septos. Provavelmente, esta mudança na concentração e no número de células, possa está relacionada à diminuição dos antígenos parasitários, que naturalmente ocorre com o evoluir da infecção tanto com a morte e degradação dos vermes, quanto com a diminuição paulatina do potencial antigênico das coleções de ovos. Contudo, a explicação do porque dessas células mesmo após um ano de infecção serem vistas em alguns septos não é bem clara, ainda mais que estas células não foram objeto de estudo particularizado neste trabalho.

A fibrose hepática é caracterizada por uma aumentada deposição e alterada composição da matriz extracelular, tais como, excesso de colágenos de tipos I, III, e IV (FRIEDMAN, 1993). A fibrose e a cirrose avançadas são geralmente consideradas como sendo condições irreversíveis mesmo após a remoção do agente causador da agressão. Entretanto, o desenvolvimento da cirrose é caracterizado por uma diminuição da atividade da colagenase hepática, implicando numa crítica redução da capacidade do fígado agredido em remodelar a matriz fibrótica. Contudo, dados disponíveis na literatura mostram que em fibroses e cirroses hepáticas humanas, causadas pela

obstrução biliar, infecções virais crônicas e deposição de metais, o tratamento depois de retirado o agente causador se acompanha de redução significativa do colágeno depositado excessivamente, fato confirmado em alguns modelos experimentais de fibrose hepática (ABDEL-AZIZ et al., 1990; ROJKIND & DUNN, 1979; HUNT, 1992;). Segundo IREDALE e colaboradores (1998), a fibrose mesmo em estágios avançados pode ser remodelada. Para comprovarem a sua hipótese, eles trataram ratos duas vezes por semana com CCl_4 (por via intraperitoneal) e suspenderam o tratamento após quatro semanas. Em seguida, sacrificaram os ratos em pontos pré-estabelecidos (0, 3, 7 e 28 dias após a suspensão do tratamento). Os autores sugerem que o evento chave no processo de resolução espontânea da fibrose é a perda de células estreladas ativadas através do processo de apoptose. Ainda segundo os autores, a fibrose está associada a um aumento na expressão de células positivas para α-actina de músculo liso e para desmina. Eles têm mostrado adicionalmente que esta mudança está associada com um aumento paralelo na taxa de apoptose de células não-parenquimais. Contudo, o restabelecimento espontâneo do estado de fibrose hepática é acompanhado pela perda de células estreladas ativadas α-actina de músculo liso positivas através de apoptose. Neste experimento, os autores verificaram figuras apoptóticas em clara associação com o septo fibrótico ou com os resíduos de septos fobróticos. Por imunohistoquímica, demonstraram que as células nessas áreas são sempre exclusivamente α-actina de músculo liso positivas e, por dupla marcação, demonstraram através da técnica do TUNEL núcleos condensados positivos dentro das faixas fibróticas que são observados dentro das células α-actina de músculo liso positivas, indicando que a

apoptose observada nessas mesmas regiões está ocorrendo em células estreladas ativadas. Um outro aspecto importante verificado pelos autores foi a confirmação da diminuição do conteúdo de hidroxiprolina tecidual comprovado pela redução nos níveis do colágeno durante o período de restabelecimento.

Nas fases mais tardias, da infecção, seis meses a um ano (Manuscrito II), observamos, através da coloração com o sírius-vermelho, que os septos apresentavam-se mais escassos e mais delgados que aos três meses, demonstrando um claro sinal de regressão espontânea da fibrose que, foi se acentuando com o passar do tempo. Contudo, a fibrose não desapareceu completamente, pois, o padrão septado do fígado se manteve mesmo na ausência dos estímulos antigênicos do início (vermes e ovos ativos). As coleções de ovos presentes, provavelmente, não possuíam mais o mesmo potencial antigênico das fases mais precoces, pois, os mesmos, demonstravam, claramente estar menos ativos. Esta atividade pode ser avaliada nas lâminas representativas de infecções antigas, coradas com sírius-vermelho, nas quais verificamos a ínfima presença das fibras colágenas em torno das coleções de ovos, quando comparadas com a exuberância de fibras encontradas nas lâminas representativas das fases mais precoces da infecção.

A avaliação dos níveis de anticorpos (Manuscrito II) em animais com 3, 6 e 12 meses de infecção contra os ovos não-embrionados da *C. hepatica* demonstraram títulos comparáveis, não havendo diferenças significativas ($p>0,05$). Quando foram analisados os níveis de anticorpos dos mesmos grupos contra os vermes adultos, a resposta foi bem semelhante. Esses

achados nos levam a concluir que a resposta humoral não deve está expressando o que se passa *in situ*, pois, a partir dos seis meses, comprovadamente, não encontramos mais vestígios de vermes no parênquima e os ovos que alí permanecem por todo o tempo, aprisionados, provavelmente, encontram-se num estágio de reduzida ou nenhuma antigenicidade. Quando testados, estes ovos não embrionaram.

A avaliação da dosagem da hidroxiprolina realizada nos animais com 3, 6, 9 e 12 meses pós-infecção demonstrou uma redução significante da fibrose ($p<0,05$) quando se comparou os animais com três meses de infecção com os demais animais representantes de cada grupo. Esses dados confirmam as nossas observações feitas a partir da análise das lâminas coradas com sírius-vermelho nas quais pudemos notar nítida redução dos septos fibrosos com o evoluir da infecção o que nos indicou que poderia estar havendo um processo de remodelamento da fibrose. Contudo, após os seis meses de infecção o conteúdo da hidroxiprolina foi praticamente o mesmo, apontando para uma estabilização no processo de degradação do colágeno.

Os componentes da matriz extracelular como os colágenos de tipos I e III estiveram presentes dentro do septo fibroso em todos os estágios observados (Manuscrito II). O colágeno de tipo IV e a laminina que são marcadores da membrana basal expressaram a rica vascularização do septo fibroso e também a sua persistência com o passar do tempo. A fibronectina foi sendo cada vez menos expressa, no septo, ao longo da infecção quando comparada com os outros elementos. Esses achados demonstram que apesar da clara diminuição dos elementos matriciais, a fibrose hepática persiste como um sinal, irrefutável, dos rearranjos vasculares pelos quais

passou o parênquima hepático. Dessa forma, a fibrose septal hepática, nesse modelo, parece funcionar como mero suporte dos novos vasos formados como resultado da agressão e adaptação sofrida pelo fígado. Assim sendo, a fibrose septal atua como estroma para os vasos sangüíneos conduzindo o sangue portal para os sinusóides.

Durante o processo evolutivo, alguns mamíferos entre eles, o urso polar, o racum, o camelo, o porco selvagem, o porco doméstico e o dromedário, desenvolveram um padrão hepático diferente, septado, porém, considerado, funcionalmente, como normal (EKATAKSIN & WAKE, 1991; MACSWEEN et al., 2002). Nessas espécies, o fígado apresenta um aspecto trabeculado diferente do que se observa nos demais mamíferos inclusive, no Homem. (EKATAKSIN & WAKE, 1991). O interessante é que, a septação fibrosa pode ser induzida experimentalmente e o fígado pode permanecer sem elevação da pressão portal ou mesmo, ter prejudicado a sua função hepática. A fibrose septal, difusa, induzida no fígado de murinos por soro heterólogo (eqüino ou porcino) não mostra nenhum sinal de distúrbio microcirculatório ou dano hépato-celular (RUBIN et al., 1968; SHIBAYAMA,et al., 1992). Visto que, o septo fibroso subdivide o parênquima em pseudolóbulos, similares aos lóbulos hepáticos do porco (BHUNCHET & WAKE, 1998), o fígado lobulado retém essencialmente a capacidade e tendência para servir a circulação normal indiferente do aspecto rígido da septação (BHUNCHET & FUJIEDA, 1993). Por outro lado, a septação esporádica que ocorre de forma irregular caracteriza os fígados com hipertensão portal idiopática (LUDWIG et al., 1993a; LUDWIG et al. 1993b), alguns com hipertensão portal não-cirrótica (NAKANUMA et al., 1996) e a

cirrose septal incompleta (BARNETT et al., 1992). Esse padrão trabeculado, aspecto normal nas espécies anteriormente citadas, assemelha-se morfologicamente ao que observamos no fígado do rato infectado por *C. hepatica*. Contudo, ainda não sabemos, com precisão, porque este padrão se desenvolve nesses ratos.

Ao analisarmos comparativamente o perfil das enzimas hepáticas dos animais (Manuscrito II), com 3, 6, 9 e 12 meses de infecção, verificamos não haver qualquer indicativo de alteração ou disfunção da função hepática (dados insuficientes para análise estatística), o que talvez, seja explicado pela distribuição dos septos de tecido conjuntivo no parênquima subdividindo-os em pseudos-lóbulos, similares aos lóbulos hepáticos do porco como sugerido por BHUNCHET & WAKE, (1998), e que essa angioarquitetura, mantenha, sem alteração, a dinâmica da microcirculação hepática mesmo nas fases mais tardias onde ocorre uma regressão espontânea da fibrose e a permanência de alguns septos incompletos espalhados pelo parênquima. Provavelmente, as alterações vasculares promovidas nas fases iniciais da infecção não sofram modificações, mais tardiamente, devido ao equilíbrio microcirculátório estabelecido.

6 CONCLUSÕES

1. A fibrose hepática septal da capilaríase inicia-se entre o $25^{\underline{0}}$ e o $27^{\underline{0}}$ dias após a infecção do rato.
2. A fibrose se inicia, principalmente, a partir dos espaços porta e, em alguns casos, a partir da cápsula fibrosa formada em torno dos parasitas ou da cápsula do fígado.
3. Os septos mais precoces originados dos espaços porta são formados predominantemente por células endoteliais e fibroblastos proliferados, com poucas células α-actina positivas.
4. Os vasos neoformados a partir dos espaços porta avançam para a zona III do lóbulo, acompanhados da deposição de feixes de colágeno.
5. A proliferação endotelial e fibroblástica precoce é acompanhada de aumento de expressão de TGF-β, TGF-βR e PDGF nas células dos septos em formação.
6. A partir dos seis meses de infecção começa ocorrer um processo de regressão parcial da fibrose, o qual logo se estabiliza.
7. Septos fibrosos permanecem após 1 ano ou mais de infecção, em associação com os vasos que brotaram a partir dos espaços porta, demonstrando que a fibrose persistente é apenas a fibrose perivascular, de sustentação.
8. Os vasos neoformados nos septos persistem, lançando o sangue nos sinusóides, sem que isso represente fator de alteração da função

hepática, que estava normal durante toda a observação, como indicado pela avaliação clínica e laboratorial.

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8 ANEXOS

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INTERSTITIAL DENDRITIC CELLS OF THE HEART HARBOR *TRYPANOSOMA CRUZI* ANTIGENS IN EXPERIMENTALLY INFECTED DOGS: IMPORTANCE FOR THE PATHOGENESIS OF CHAGASIC MYOCARDITIS

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Abstract. Heart sections from 16 mongrel dogs, two normal controls and 14 infected with *Trypanosoma cruzi*, were submitted to immunohistochemical staining with either rabbit anti-cow S100 Protein monoclonal antibody or rabbit anti-*T. cruzi* purified specific antibody, using the peroxidase technique to investigate the participation of the interstitial dendritic cells of the heart (IDCs) in myocarditis of Chagas disease. *Trypanosoma cruzi* antigens were revealed as granular and dense deposits in IDC membrane in the heart of infected dogs both during acute and chronic myocarditis, but not in normal controls. Anti-S100 Protein labeled the IDCs, both in normal and infected dogs and a significant increase in the numbers of IDCs occurred in the myocardium, proportionally to the intensity of the inflammatory infiltration. These findings suggest that IDCs, probably by presenting *T. cruzi* antigens to immune-competent cells, play an important role in the pathogenesis of Chagas disease.

INTRODUCTION

Interstitial dendritic cells (IDCs) of the myocardium were first described by Hart and Fabre.[1] Immunohistochemistry with anti-MHC class II antibodies intensely stains these cells. Interstitial dendritic cells also exhibit the ability to bind to antigens and to stimulate T lymphocyte responses.[2] They are recognized as specialized antigen-presenting cells that initiate immune response and can eventually migrate to the spleen, where they may have a significant role during heart transplant rejection.[3] Migration of IDCs to the T-cell zone of the spleen may give rise to continuous sensitization of the heart to a delayed type hypersensitivity, the kind of immune reaction presently considered to be the main pathogenetic mechanism involved in Chagas disease myocarditis.[4]

Although a great deal of attention has been paid to the participation of several cell types in the pathogenesis of Chagas myocarditis, a role for IDCs has not yet been considered. Evidently, macrophages, lymphocytes, plasmocytes, as well as fibroblasts play important roles during host reactions against *Trypanosoma cruzi* antigens. The predominance of different subsets of lymphocytes, CD4 or CD8, which seems variable accordingly to the phase of the infection, has been thoroughly investigated.[5–7] However, whether myocardial IDCs play a role in this process has not been established. Therefore, the present study was designed to investigate the participation of IDCs in Chagas myocarditis, not only to complement data on cellular types involved in this inflammatory process, but also to further investigate the pathogenesis of myocarditis.

MATERIALS AND METHODS

Sixteen mongrel dogs were used in the present investigation: 14 experimentally infected with *T. cruzi* either in the acute or chronic phases of infection and two normal controls. Maintenance and care of experimental animals complied with the Centro de Pesquisas Gonçalo Moniz/Fiocruz guidelines for humane use of laboratory animals.

Acute phase. Six dogs weighing 1,100 to 3,200 kg were infected with the 12 SF strain (1×10^5 trypomastigotes/kg of body weight [b.w.]) or the 21SF strain of *T. cruzi*, (4×10^5 trypomastigotes/kg body weight), administered intraperitoneally (Table 1). Both parasite strains had previously been isolated from human cases of acute Chagas disease from São Felipe, BA, Brazil and characterized according to their biological behavior as biodeme Type II and their isoenzymic patterns as zymodeme Z2.[8] Animals were examined daily. Parasitemic levels were evaluated weekly in peripheral blood drawn from the cephalic vein by venipuncture, and by counting the number of trypomastigotes in 50 microscopic fields (× 400). Pentobarbital sodium was used to kill the animals and complete autopsies were performed. Portions of the heart were fixed in paraformaldehyde and cryopreserved. Other portions of the heart were fixed in buffered 10% formalin, paraffin embedded, and sections used for histopathological or immunohistochemical techniques.

Chronic infected phase. Paraffin blocks of heart tissue from eight dogs were taken from the files. General data concerning inocula, duration of infection and histopathology, are presented in Table 2. Three dogs were infected with the Colombian strain, which was isolated from a human case in Colombia, and previously characterized by the biological characters as biodeme Type III and by the isoenzymic profils as zymodeme Z1[8] (Inoculum: 5×10^4 blood forms/kg b.w.) Five dogs belonging to two groups, were infected with the 12SF strain, being 3 with 6×10^4 and 2 with 1×10^5 blood forms/kg b.w. These cases were representative of 1) 5 dogs which presented diffuse chronic myocarditis following repeated treatment with low dose cyclophosphamide (Cy), as described elsewhere by Andrade and others;[9] 2) one dog with spontaneous chronic *T. cruzi* myocarditis; and 3) two dogs sacrificed during the chronic indeterminate phase of infection.

Normal controls. Two healthy uninfected adult dogs were maintained in separate quarters, but under the same general conditions as the infected groups, and used as controls.

Immunohistochemical staining of interstitial dendritic cells of the heart. Immunohistochemical staining for IDCs was performed in 5 micron-thick cryostat sections obtained from cryopreserved tissues, as well as in 5 micron-thick sections from paraffin blocks. Either one of two primary antibodies were used: a commercially purchased monoclonal

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Chronic infected phase. Paraffin blocks of heart tissue from eight dogs were taken from the files. General data concerning inocula, duration of infection and histopathology, are presented in Table 2. Three dogs were infected with the Colombian strain, which was isolated from a human case in Colombia, and previously characterized by the biological characters as biodeme Type III and by the isoenzymic profils as zymodeme Z1[8] (Inoculum: 5×10^4 blood forms/kg b.w.) Five dogs belonging to two groups, were infected with the 12SF strain, being 3 with 6×10^4 and 2 with 1×10^5 blood forms/kg b.w. These cases were representative of 1) 5 dogs which presented diffuse chronic myocarditis following repeated treatment with low dose cyclophosphamide (Cy), as described elsewhere by Andrade and others;[9] 2) one dog with spontaneous chronic *T. cruzi* myocarditis; and 3) two dogs sacrificed during the chronic indeterminate phase of infection.

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Immunohistochemical staining of interstitial dendritic cells of the heart. Immunohistochemical staining for IDCs was performed in 5 micron-thick cryostat sections obtained from cryopreserved tissues, as well as in 5 micron-thick sections from paraffin blocks. Either one of two primary antibodies were used: a commercially purchased monoclonal

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INTERSTITIAL DENDRITIC CELLS OF THE HEART HARBOR *TRYPANOSOMA CRUZI* ANTIGENS IN EXPERIMENTALLY INFECTED DOGS: IMPORTANCE FOR THE PATHOGENESIS OF CHAGASIC MYOCARDITIS

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Abstract. Heart sections from 16 mongrel dogs, two normal controls and 14 infected with *Trypanosoma cruzi,* were submitted to immunohistochemical staining with either rabbit anti-cow S100 Protein monoclonal antibody or rabbit anti-*T. cruzi* purified specific antibody, using the peroxidase technique to investigate the participation of the interstitial dendritic cells of the heart (IDCs) in myocarditis of Chagas disease. *Trypanosoma cruzi* antigens were revealed as granular and dense deposits in IDC membrane in the heart of infected dogs both during acute and chronic myocarditis, but not in normal controls. Anti-S100 Protein labeled the IDCs, both in normal and infected dogs and a significant increase in the numbers of IDCs occurred in the myocardium, proportionally to the intensity of the inflammatory infiltration. These findings suggest that IDCs, probably by presenting *T. cruzi* antigens to immune-competent cells, play an important role in the pathogenesis of Chagas disease.

INTRODUCTION

Interstitial dendritic cells (IDCs) of the myocardium were first described by Hart and Fabre.[1] Immunohistochemistry with anti-MHC class II antibodies intensely stains these cells. Interstitial dendritic cells also exhibit the ability to bind to antigens and to stimulate T lymphocyte responses.[2] They are recognized as specialized antigen-presenting cells that initiate immune response and can eventually migrate to the spleen, where they may have a significant role during heart transplant rejection.[3] Migration of IDCs to the T-cell zone of the spleen may give rise to continuous sensitization of the heart to a delayed type hypersensitivity, the kind of immune reaction presently considered to be the main pathogenetic mechanism involved in Chagas disease myocarditis.[4]

Although a great deal of attention has been paid to the participation of several cell types in the pathogenesis of Chagas myocarditis, a role for IDCs has not yet been considered. Evidently, macrophages, lymphocytes, plasmocytes, as well as fibroblasts play important roles during host reactions against *Trypanosoma cruzi* antigens. The predominance of different subsets of lymphocytes, CD4 or CD8, which seems variable accordingly to the phase of the infection, has been thoroughly investigated.[5–7] However, whether myocardial IDCs play a role in this process has not been established. Therefore, the present study was designed to investigate the participation of IDCs in Chagas myocarditis, not only to complement data on cellular types involved in this inflammatory process, but also to further investigate the pathogenesis of myocarditis.

MATERIALS AND METHODS

Sixteen mongrel dogs were used in the present investigation: 14 experimentally infected with *T. cruzi* either in the acute or chronic phases of infection and two normal controls. Maintenance and care of experimental animals complied with the Centro de Pesquisas Gonçalo Moniz/Fiocruz guidelines for humane use of laboratory animals.

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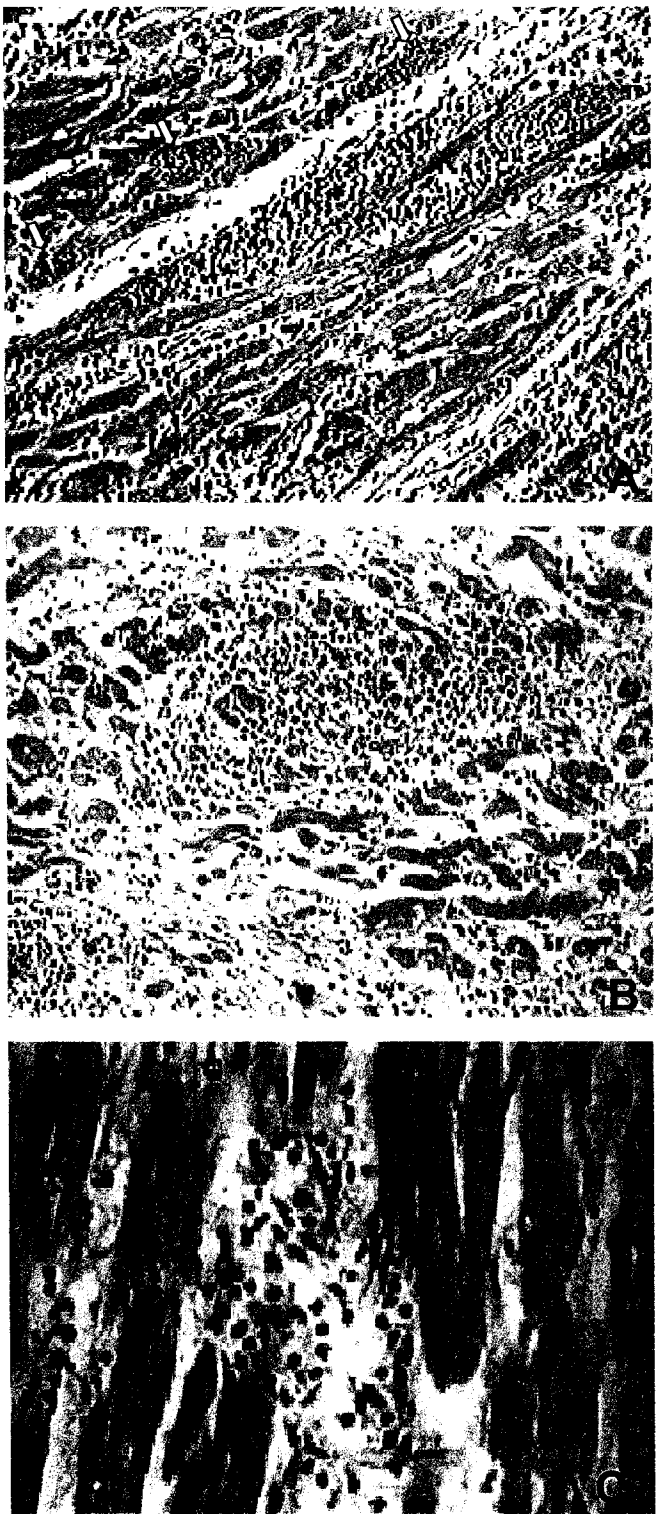

FIGURE 1. Histopathological aspects of the myocardium. **A.** Acute myocarditis with intense mononuclear inflammatory infiltration, focal destruction of myocytes and presence of intracellular amastigotes (arrows). (Hematoxylin and Eosin × 250). **B.** Chronic diffuse myocarditis with focal destruction of cardiac myocytes; focal and diffuse mononuclear inflammatory infiltration and interstitial fibrosis. (Hematoxylin and Eosin × 250). **C.** Chronic indeterminate form, showing mild focal myocarditis. (Hematoxylin and Eosin × 400).

TABLE 1

General data on dogs in the acute phase of infection with *Trypanosoma cruzi*

Case no.	Identification no.	*T. cruzi* strain	Initial weight (g)	Inoculum* (Tryp/kg/b.w.)	Duration (days)	Degree of infiltration
1	135	12 SF	2,200	1×10^5	25	+++
2	173	21 SF	1,100	4×10^5	26	+++
3	183	21 SF	2,500	4×10^5	34	+++
4	184	21 SF	2,900	4×10^5	34	+++
5	185	21 SF	2,500	4×10^5	32	+++
6	190	21 SF	3,200	4×10^5	34	+++

* Inoculum = Number of trypomastigotes/kg/body weight.
+++ = intense and diffuse inflammatory infiltration.

anti-cow S100 Protein (for the identification of IDCs) and a purified specific anti-*T. cruzi* IgG obtained in rabbits. Anti-S100 Protein is the purified IgG fraction of the antiserum produced in rabbits by injections with purified S-100 protein from bovine brain. It was used in the present investigation, because anti-dendritic antibodies specific for dogs are not yet available and since S100 protein is present in IDCs and can be labeled with the anti-S100 protein.[10,11]

Technical procedures. 1) Cryopreserved sections: fragments of the heart were fixed in a solution made of 4% paraformaldehyde, 0.1M cacodylate, and 7.5% sacharose, for 8 to 12 hr at 4°C. Following fixation, fragments were washed with 0.1M cacodylate + 0.2M (pH 7.4) sacharose, for 12 hr at 4°C. Tissues were embedded in Tissue Teck and cryopreserved in liquid nitrogen. 2) Paraffin sections were treated with 100% xylene (2 baths of 15 min) followed by acetone for 10 min. Sections were hydrated in decreasing concentrations of ethanol in water and endogenous peroxidase was blocked with a solution of hydrogen peroxide (0.3%) and methanol. Digestion was performed with a solution of trypsin (Sigma) at 0.1% with $CaCl_2$ and NaCl, pH 7.6 for 45 min. Both deparaffinized and cryostat sections were submitted to the same procedures of immunostaining: sections were washed in phosphate buffer (PBS) pH 7.4; non-specific reactions were blocked with 10% non-fat dry milk in PBS for 15 min.

S100 Protein immunolabeling. Incubation took place for 1 hr at 37°C in an humidified chamber with rabbit anti-cow S100 Protein monoclonal antibody (DAKO) diluted at 1:100 in PBS plus 2% Tween 20 and 1% bovine serum albumin. This was followed by treatment with normal serum during 20 min at room temperature; incubation with secondary biotinylated goat anti-rabbit antibody (Vectastain Elite ABC kit rabbit IgG, Vector Lab PK6101), in the dilution of 1:20, during 30 min at 37° C and incubation with Vectastain ABC reagent in PBS in humidified chamber at 37° C; the color was developed with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 at room temperature. Sections were counterstained with 1% methyl-green for 2 min, dehydrated and mounted with Permount.

Trypanosoma cruzi antigen immunolabeling. Sections were treated with purified, specific anti-*T.cruzi* IgG produced in rabbits, in the dilution of 1:640 followed by biotinylated goat anti-rabbit IgG antibody (Vectastain Elite ABC kit, PK6101), following the same steps described above. Anti-*T. cruzi* antibodies were produced by two rabbits immunized with whole *T. cruzi* antigen extracted from culture forms of

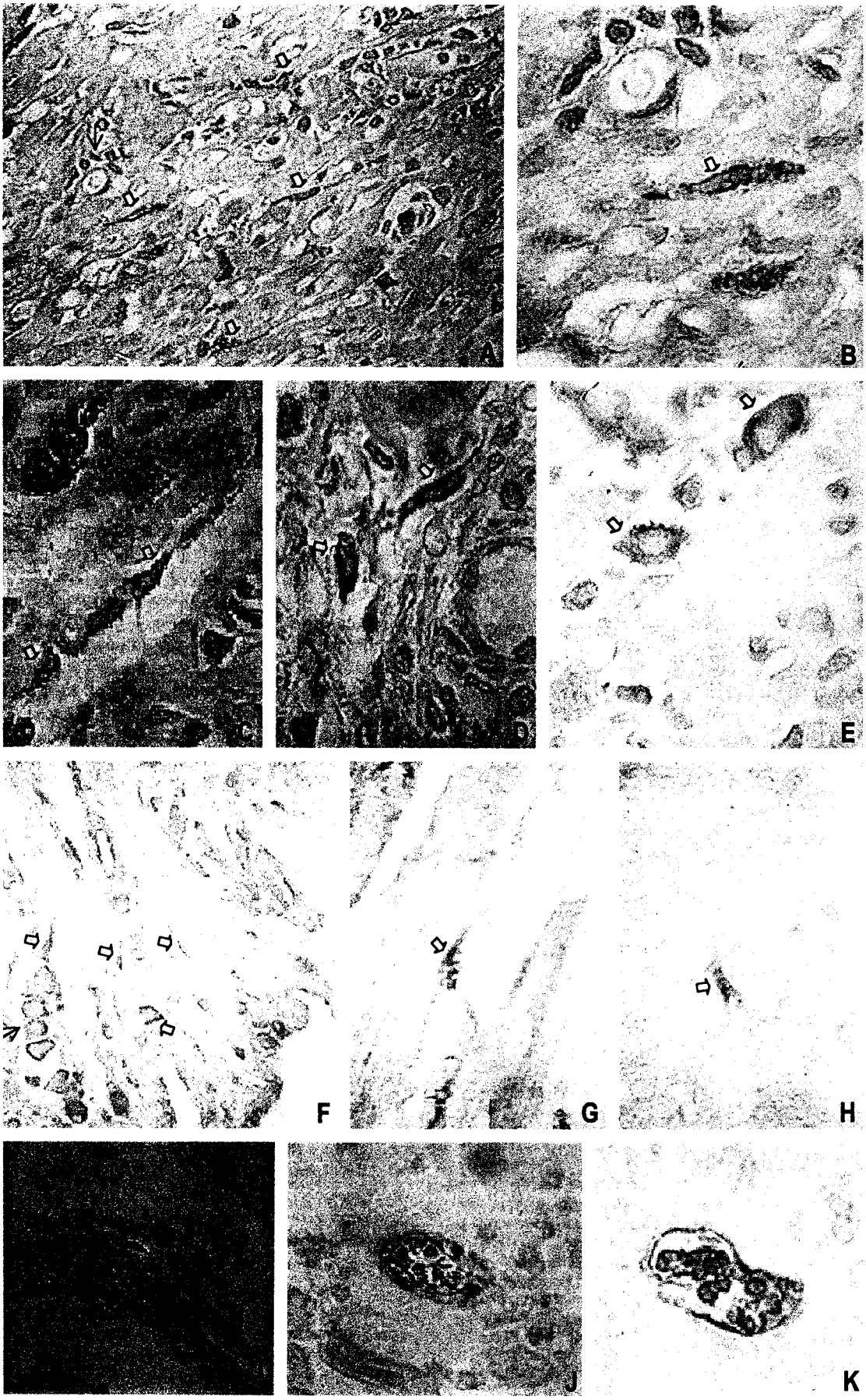
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TABLE 2
General data on dogs chronically infected with *Trypanosoma cruzi*

Case no.	Identification no.	Infection (days)	*T. cruzi* strain	Inoculum (Tryp/kg/b.w.)	Cyclophosphamide*	Myocarditis
7	74	338	Colombian	5×10^4	Yes	CRDM
8	75	338	Colombian	5×10^4	Yes	CRDM
9	82	338	Colombian	5×10^4	Yes	CRDM
10	86	122	12 SF	6×10^4	Yes	CRDM
11	87	122	12 SF	6×10^4	Yes	CRDM
12	112	213	12 SF	1×10^5	No	MFM
13	115	213	12 SF	1×10^5	No	MFM
14	129	94	12 SF	6×10^4	No	CRDM

Inoculum = Number of trypomastigotes (blood forms) /kg body weight.
* Treatment with low doses of Cyclophosphamide.
CRDM = chronic diffuse myocarditis; MFM = mild focal myocarditis (indeterminate form).

the parasite, obtained from Warren culture medium (Bacto Brain Heart Infusion, DIFCO Laboratories, Detroit, MI). Parasites were washed three times in PBS, pH 7.2, submitted successively to freezing in liquid nitrogen and thawing, and mixed in complete Freund's adjuvant. Serum tested by indirect immunofluorescence showed titers of 1:640 specific for *T. cruzi*. Gamma globulin was precipitated with ammonium sulphate, dialysed in PBS, pH 7.0, and purified a second time using Sephadex for separation of IgG antibodies. A third purification was performed in CnBr sepharose with *T. cruzi* antigen. The eluate containing the specific antibodies was concentrated with polyethylene-glycol.

Quantitative evaluation of IDCs in the immunostained heart sections. The number of IDCs was counted in 5 microscopic fields, not successively, using an ocular 10× and an objective 40×. Counting was performed on paraffin sections of heart tissue from control and infected dogs during acute and chronic infections. Statistical analysis of results was performed by unpaired *t*-test. The mean and standard deviation (SD) was established for each case and the number of cells/mm^2 calculated. The mean (± SD) of the measurements obtained for each group in sections of the heart (either acute and chronic infection), immunolabeled with either anti-S100 Protein or anti-*T. cruzi* antibodies, were compared with normal controls labeled with anti-S100 Protein. Comparison was also made between the groups of chronic diffuse myocarditis (CRDM) and the indeterminate form with mild focal myocarditis (MFM).

TABLE 3
Interstitial dendritic cells (IDCs) in normal control dog hearts

Normal controls	Number of IDCs pmm^2 anti-S100 protein
C1	29.8 ± 14.5
C2	44.8 ± 10.8
Mean	37.30 ± 7.5

C1 = control no. 1; C2 = control no. 2.

RESULTS

Non-infected control dogs showed hearts histologically within normal limits. Interstital dendritic cells were demonstrated by immunohistochemistry with anti-S100 Protein antibodies in the interstitial tissue of the normal myocardium. Cells were elongated, isolated, and uniformly distributed with scanty, compact cytoplasm, a central, round, pale nucleus, and two or three fine cytoplasmic processes with bi- or tripolar extensions, which sometimes gave the cell a fusiform or triangular appearance.

For the dogs with acute *T. cruzi* infection, intense myocarditis was present in all cases, as shown in Table 1, characterized by intense and diffuse inflammatory infiltration (grade +++), more prominent in the atrial wall (Figure 1A). Variable degrees of damage in parasitized and non-parasitized myocells associated with diffuse and focal mononuclear and also focal polymorphonuclear infiltration were present, as previously described.[12] During the chronic indeterminate stage of infection, a mild focal myocarditis was present in two dogs that had not been treated with Cy (Table 2). Cardiac lesions were rare, with small mononuclear inflammatory infiltrates involving the interstitial myocardium and subendocardium as shown in Figure 1C. Previous description of these infiltrates by electron microscopy has shown evidences of apoptosis of lymphocytes, macrophages, and plasmocytes, determining a self-control of inflammation in this phase.[13]

A chronic diffuse myocarditis (Table 2) was present in dogs chronically infected with *T. cruzi* and treated with low dose Cy,[9] characterized by intense inflammatory infiltration (Figure 1B), and focal damage of myocardiocytes. In one non-Cy-treated dog a CRDM was also seen; this case represents an early evolutive chronic myocarditis, 94 days post-infection. Two types of heart lesions were present: focal destruction of cardiac myocells with scattered foci of mono-

←

FIGURE 2. Sections of the heart of dogs infected with *Trypanosoma cruzi*, submitted to immunohistochemistry staining with peroxidase and revealed with diaminobenzidine (DAB), for immunolabeling of interstitial dendritic cells (IDCs), using as primary antibodies either anti-S100 Protein or anti-*T. cruzi* purified antibodies: **A, B, C,** and **D.** Acute myocarditis: IDCs appear labeled with anti-*T. cruzi* antibodies, with its peculiar morphology and localization, in the interstitium and associated with inflammatory infiltration. **A.** General view showing several IDCs revealing the presence of *T. cruzi* antigens on the membrane (arrows) in the presence of lymphocytes infiltration (thin arrow) (× 400). **B.** Higher magnification of one cell (white arrow) in **A.** showing dense granular, deposits in the membrane (× 1,000). **C.** and **D.** IDCs labeled with anti-*T. cruzi* antibody, related to the inflammatory infiltrates (× 1,000). **E.** Acute myocarditis with macrophages exhibiting *T. cruzi* antigens in the cytoplasm. **F.** Chronic myocarditis in a dog treated with cyclophosphamide (Cy): several IDCs are labeled with anti-S100 protein (arrows), showing their typical morphology and localization; lymphocytic infiltration (thin arrow) is present (× 1,000). **G.** Chronic indeterminate form, presence of IDC-positive for *T. cruzi* antigen, in a dog not treated with Cy (× 1,000). **H.** Preserved area of the myocardium in a dog treated with Cy, that presented a chronic myocarditis, showing isolated IDCs, with positive immunolabeling for anti-*T. cruzi* antibodies (× 1,000). **I.** Section of the heart of a normal control dog, showing IDC-positive staining with anti-S100 Protein (× 400). **J.** Acute infection. Intracellular amastigotes with positive nuclear and kinetoplast reaction for anti-S100 Protein but the parasite membrane is negative (× 400). **K.** Acute infection. Anti-*T. cruzi* antibodies gave a coarse granular cytoplasmic staining of amastigotes and clearly depicted their external membrane and kinetoplast (× 1,000).

TABLE 4

Quantitative evaluation of interstitial dendritic cells (IDCs) in the heart of dogs infected with *Trypanosoma cruzi*

Acute infection			
		No. of dendritic cells/mm²*	
Case no.	Identification no.	Anti-S100 protein	Anti-*T. cruzi*
1	135	83.1 ± 18.4	63.9 ± 34.3
2	173	79.2 ± 13.9	73.8 ± 27.2
3	183	106.9 ± 21.0	83.7 ± 14.4
4	184	97.6 ± 21.5	80.9 ± 21.5
5	185	98.0 ± 15.6	92.3 ± 31.7
6	190	79.5 ± 9.2	92.3 ± 23.5
	Mean ± SD	90.7 ± 4.7	81.1 ± 4.5
	Normal Control	37.3 ± 7.5	
Statistical Analysis†		$P = 0.0013$‡	$P = 0.0027$§

* Mean ± SD.
† Comparison of means.
‡ Anti-S100 protein × normal controls.
§ Anti-*T. cruzi* × normal controls. Unpaired *t*-test (*P* value < 0.05).

nuclear inflammatory infiltration and diffuse mononuclear cells infiltration with focal concentrations in the peri-vascular space and fibrous thickening of the interstitial matrix.

Identification of IDCs was possible by immunostaining with the anti-S100 Protein monoclonal antibody. In sections treated with anti-*T. cruzi* antibodies, IDCs appeared strongly stained (Figures 2 A–D, G, and H) and with their characteristic morphological features, as seen when anti-S100 Protein antibody was used (Figure 2F). Not only the morphological appearance but also the distribution of the IDCs was similar in sections treated either with anti-S100 Protein or anti-*T. cruzi* antibodies. However, IDCs in the hearts of normal controls failed to stain with anti-*T. cruzi* antibodies. In the presence of myocarditis, IDCs were seen to be more concentrated around areas of focal myocardial infiltrations and near parasitized cardiac myocells, forming clusters of three or more elongated and characteristic dendritic cells associated with lymphocytes (Figures 2A, C, and D). In the preserved areas of the myocardium, IDCs were rare and appeared isolated in the interstitium on the myocyte membrane (Figures 2G and H). Besides the IDCs, macrophages in the infiltrates occasionally presented positive reaction for *T. cruzi* particulated antigens into the cytoplasm, revealed by the staining of anti-*T. cruzi* antibodies (Figure 2E).

Intracellular amastigotes showed positive nuclear and kinetoplast reaction for anti-S100 Protein, but the parasite membrane remained negative (Figure 2J). Anti-*T. cruzi* antibodies gave a coarse granular cytoplasmic staining of amastigotes and clearly depicted their external membrane (Figure 2K).

Quantitative evaluation of IDCs. Table 3 shows the quantitative evaluation of the IDCs in normal dogs. The IDCs were increased in number in Chagas myocarditis, acute or chronic diffuse, as demonstrated by quantitative analysis (Tables 4–6). Statistical analysis (considering a level of significance of $P < 0.05$ revealed significant increase in the number of IDCs in cases of acute infection, as seen in sections labeled with anti-S100 ($P = 0.0013$) or anti-*T.cruzi* ($P = 0.027$) antibodies, as compared with the normal controls (Table 4). The same was true when cases of chronic diffuse myocarditis were compared to normal controls ($P = 0.006$, for anti-S100 Protein and $P = 0.005$, for anti-*T.cruzi*) (Table 5). Comparing the number of IDCs in the indeterminate form and controls, no significant statistical difference was detected (Table 6).

TABLE 5

Evaluation of number of interstitial dendritic cells (IDCs) in the hearts of dogs in the chronic phase of infection with *Trypanosoma cruzi* that show chronic diffuse myocarditis

		Number of IDCs/mm² (mean ± SD)	
Case no.	Identification no.	Anti-S100 protein	Anti-*T. cruzi*
7	74	98.0 ± 21.5	90.8 ± 9.2
8	75	108.2 ± 26.6	76.5 ± 10.5
9	82	159.0 ± 34.8	66.0 ± 19.1
10	86	86.6 ± 10.5	61.0 ± 26.3
11	87	123.5 ± 29.8	78.1 ± 7.1
14	129	126.3 ± 63.5	84.2 ± 11.2
	Mean ± SD	116.9 ± 10.4	76.1 ± 4.5
	Controls	37.3 ± 7.5	
Statistical analysis*		$P = 0.0062$†	$P = 0.0051$‡

* Comparison of means between the chronically infected and normal controls.
† Anti-S100 Protein × normal controls.
‡ Anti-*T. cruzi* × normal controls. Unpaired *t*-test (*P* value < 0.05).

DISCUSSION

The present study demonstrates for the first time that cells morphologically identified as IDCs, can be immunohistochemically stained with specific anti-*T. cruzi* antibodies, in the interstitium of the myocardium of dogs with acute and chronic Chagas myocarditis. The significance of this finding for the pathogenesis of Chagas myocarditis seems evident, since IDCs are antigen-presenting cells to immune-competent T lymphocytes. Interstitial dendritic cells from normal animals, identified by S100 Protein-positive staining, did not react with anti-*T. cruzi* antibodies, which indicates that the staining observed in IDCs of infected animals is due to the presence of *T. cruzi* antigen(s).

In a previous study, Andrade and others[14] demonstrated the presence of *T. cruzi* antigens in the cell membrane of follicular dendritic cells of the spleen, using immunoelectron microscopy in mice with chronic *T. cruzi* infection. This finding correlates with the persistence of a positive serology in mice free of parasites by chemotherapy. Follicular dendritic cells are present in the germinal centers of lymph follicles of the spleen and are important antigen-presenting cells to lymphocyte-B memory cells. Thus, the present demonstration of *T. cruzi* antigens in IDCs of the heart, repre-

TABLE 6

Evaluation of number of interstitial dendritic cells (IDCs) in the hearts of dogs in the chronic phase of infection with *Trypanosoma cruzi* showing the indeterminate form (mild focal myocarditis)

		Number of IDCs/mm² (mean ± SD)	
Case no.	Identification no.	Anti-S100 protein	Anti-*T. cruzi*
12	112	56.8 ± 8.6	41.1 ± 19.6
13	115	58.2 ± 19.6	26.9 ± 9.2
Mean ± SD		57.5 ± 0.7	34.0 ± 7.1
Normal control		37.3 ± 7.5	
Statistical analysis*		$P = 0.1155$†	$P = 0.7847$‡

* Comparison of means.
† Anti-S100 Protein × normal controls.
‡ Anti-*T. cruzi* × normal controls. Unpaired *t*-test (*P* value <0.05).

sentative of lymphoid dendritic cells,[2] indicates the involvement of both types of dendritic cells in humoral (spleen) and cellular (heart) responses during the course of Chagas disease.

Considering the migratory capacity of the IDCs,[2,3] it is possible that such cells present in the myocardium can migrate to the T-cell compartment of the spleen. From there and from the myocardium proper, IDCs are able to participate in the immunological process in Chagas myocarditis, considering the role of these cells in presentation of antigens in association with MHCII products and the sensitization of T cells for the production of cytokines involved in pathogenetic mechanisms.[15–17] Recently it has been shown that IDCs express IL-12 in response to specific antigen (*Staphylococcus aureus*)[18] by interaction with T cells. It is possible that *T. cruzi* antigen stimulation of IDCs could also determine similar IL-12 response, considering that surface glycoprotein molecules from both trypomastigotes and amastigotes have been shown to induce cytokine synthesis by inflammatory macrophages: IL1, IL-6, IL-12, and TNFα.[19] The complexity of the subject does not preclude the interpretation that both IDCs and *T. cruzi* components are involved in the immunological responses in Chagas myocarditis. Characterization of the inflammatory cells involved in the different phases of infection has suggested that all the classes of lymphocytes are polyclonally activated during the acute phase with a profile of antibodies typical of CD4-T cell-dependent response, but also with the participation of CD8 lymphocytes.[20] Probably the mechanisms of delayed hypersensitivity are present from the acute phase[5] with predominance of CD4 lymphocytes. However, the importance of CD8 lymphocytes has been emphasized by Tarleton[6] in the acute phase of infection and by Zhang and Tarleton[21] in chronic infection.

Spontaneously-developed *T. cruzi* chronic myocarditis is rare in dogs. Low dose Cy transforms latent infection into a chronic progressive myocarditis as has been demonstrated by Andrade and others.[9] The influence of Cy on IDCs has been shown by Limpens and others[22] with an enhancement of accessory cell function for interdigitating cells of lymph nodes. Quantitative evaluation of myocardial IDCs revealed an increased number in acute and chronic myocarditis, when compared with the normal controls or the indeterminate form of disease suggesting a direct relationship between the number of IDCs and the intensity of the inflammatory infiltration.

In rats with experimental myocardial infarction, Zhang and others[23] registered an increase in the number of IDC per mm^2 in the border of the infarcted zone and suggested that this increase is due to the recruitment of dendritic cells from bone marrow. In the acute phase, chemotatic stimuli that originated from the parasitism of cardiac cells and their rupture, with liberation of antigenic products, can be considered to be an important factor for the increased number of dendritic cells in the myocardium of dogs infected with *T. cruzi*. However, the presence of apoptosis of these cells in this phase, occurring together with the apoptosis of the inflammatory cells, myocytes, and parasites, has also been detected.[24] Apoptosis of the IDCs could be a cause of failure of antigen presentation in this phase of infection.

Parasite antigens have been identified in focal inflammatory infiltrates of chronic Chagas myocarditis, both in mice[25] and in humans.[26,27] However, the diffuse, evolutive, fibrotic, and inflammatory lesions seen in Chagasic myocarditis, compatible with a cell-mediated immune response, probably involves a continuous stimulation of cellular immunity, through the presentation of parasite antigens by IDCs to T lymphocytes.

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ARTIGO

Reinfecções e desenvolvimento da fibrose periportal esquistossomótica no modelo murino

Reinfections and the development of schistosomal periportal fibrosis in the murine model

Antonio Benigno Araújo Santos, Marcia Maria de Souza e Zilton A. Andrade

Resumo *A fibrose periportal esquistossomótica observada no modelo murino apareceu muito mais freqüentemente (69,2%) em camundongos submetidos à múltiplas infecções pelo* Schistosoma mansoni *do que naqueles animais com infecção única (11,1%). A contagem dos ovos depositados no fígado não diferiu significativamente nos dois grupos ao término dos experimentos. Embora não tenha ficado esclarecido o motivo pelo qual as infecções repetidas favorecem o desenvolvimento da fibrose periportal esquistossomótica, os dados observados fornecem apoio experimental às observações clínico-epidemiológicas que sugerem ter as reinfecções um papel na patogenia da forma hepato-esplênica da esquistossomose.*

Palavras-chaves: *Fibrose periportal. Esquistossomose. Reinfecções.*

Abstract *Experimental* pipestem *fibrosis of the liver developed more frequently (69.2%) in mice submitted to repeated infections with* Schistosoma mansoni, *than with single infection (11.1%). The counting of eggs in the liver revealed no significant differences between the two experimental groups. Although the reason why multiple infections favor the development of* pipestem *fibrosis has not been elucidated, the data obtained represent an experimental support to clinico-epidemiological claims that repeated infections play a role in pathogenesis of hepatosplenic schistosomiasis.*

Key-words: *Periportal fibrosis. Schistosomiasis. Reinfections.*

Os pesquisadores com experiência em trabalhos de campo têm suspeitado que as reinfecções pelo *Schistosoma mansoni* representam um fator adicional de importância no desenvolvimento da forma hepato-esplênica da doença. A base desta suspeita deriva da observação de que os indivíduos infectados que migram para fora da área endêmica não mais desenvolvem a forma hepato-esplênica[5,11,12]. Os que já estão com esta forma da doença têm melhor prognóstico quando fora da área endêmica e, quando tratados ou quando a transmissão é interrompida, têm mais chances de regressão do que os que permanecem em áreas de transmissão ativa[4].

Alguns dados da patologia experimental aparentemente apoiam esta tese, pois o desenvolvimento da chamada fibrose *pipestem* no chimpanzé só foi obtida com infecções múltiplas[16]. Há também um dado experimental indicativo de que os granulomas nos camundongos reinfectados são maiores do que naqueles com infecção única[9].

Um modelo mais adequado para se reproduzir a fibrose *pipestem* em camundongos foi descrito por Warren[22] em 1966. Este modelo foi mais recentemente melhor avaliado e caracterizado[1,2].

O presente estudo lança mão deste modelo para avaliar se as reinfecções pelo *S. mansoni*, resultam numa maior proporção de casos de fibrose *pipestem* do fígado, em comparação com controles submetidos à uma única infecção.

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MATERIAL E MÉTODOS

Camundongos albinos suíços, 15-18 gramas, de ambos os sexos, mantidos com dieta comercial balanceada e água *ad libitum*, foram submetidos à infecção por via transcutânea, com 30 cercárias recentemente eliminadas por diversos caramujos (*Biomphalaria glabrata*) criados e infectados em laboratório.

Sessenta dias após a exposição cercariana, com os animais eliminando ovos viáveis do *S. mansoni* nas fezes, os mesmos foram divididos ao acaso em dois grupos sendo que a metade foi simplesmente separada e mantida em observação. A outra metade foi submetida a nova exposição, com cerca de 10/15 cercárias, a cada 15 dias, perfazendo um total de 5 reinfecções. Trinta dias após a última reinfecção os animais foram sacrificados, isto é 20 semanas após o início das infecções cercarianas. Ao todo, 26 animais do grupo reinfectado e 18 controles foram examinados com a mesma metodologia descrita a seguir: sob anestesia pelo éter, o abdômen foi aberto na linha mediana e a aorta seccionada. Fragmentos do fígado foram fixados em líquido de Bouin por seis horas para exame histoiógico. Após inclusão em parafina, as secções coradas pela hematoxiiina-eosina e o pelo método do sírius vermelho para colágeno[15] foram examinadas independentemente pelos três autores, que não tinham outra informação senão um número de código escrito em cada lâmina. As poucas discordâncias que surgiram na classificação dos casos foram discutidas e resolvidas antes que as lâminas fossem decodificadas.

Para a contagem dos ovos no fígado uma parte deste órgão foi digerido em potassa segundo o método de Cheever[7].

RESULTADOS

Com a lente de campo do microscópio, as secções coradas pelo método do sírius-vermelho foram facilmente separadas, em sua maioria, em dois grupos: um que mostrava uma concentração de granulomas periovulares nas áreas periportais (Figura 1) e outro onde os granulomas apareciam como que isolados uns dos outros (Figura 2). No primeiro caso os granulomas formavam faixas mais ou menos densas de fibrose, que freqüentemente conectavam entre si vários espaços porta, deixando largas faixas do parênquima sem alterações evidentes, a não ser pela presença de pigmento enegrecido no interior de células sinusoidais. Em uns poucos casos, as duas alterações apareciam numa mesma secção. As alterações observadas aparecem na Tabela 1. A Tabela 2 mostra o resultado das contagens de ovos no fígado dos animais.

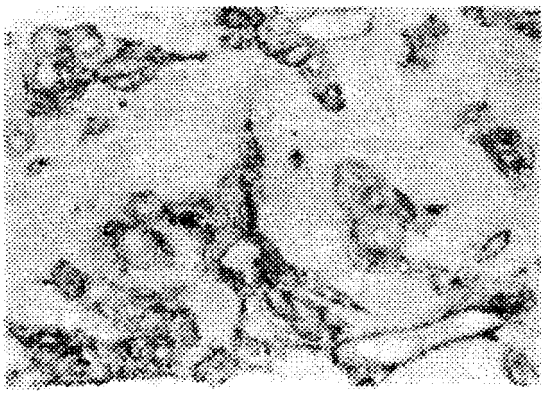

Figura 1 - Exemplo de lesão pipestem. *Os granulomas confluem nos espaços porta, associam-se com um certo grau de fibrose extragranulomatosa e formam faixas que conectam espaços porta entre si, deixando amplas áreas do parênquima livres de granulomas e com a estrutura trabecular normal do fígado. Método do sírius-vermelho para colágeno, 100x.*

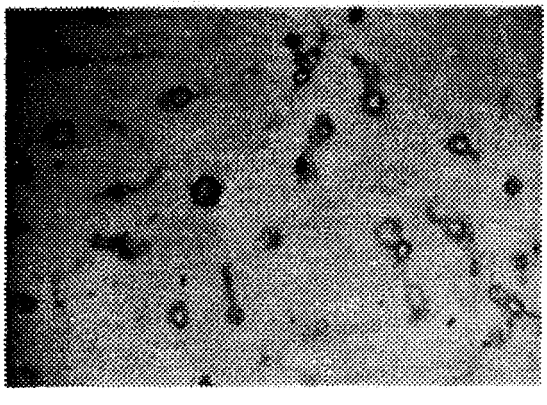

Figura 2 - Exemplo da lesão representada por granulomas isolados. *Os granulomas periovulares, isolados ou aos pares, aparecem dissociados entre si e distribuem-se ampla e esparsamente pelo tecido hepático. Método do sírius-vermelho para colágeno, 64x.*

Tabela 1 - Desenvolvimento de fibrose pipestem *em fígados de camundongos com infecção única e com múltiplas infecções pelo* Schistosoma mansoni.

Grupos	Nºanimais	c/*Pipestem*	c/Granulomas isolados	Mixtos
Reinfectados	26	18 (69,2%)	6 (23,0%)	2 (7,6%)
Controles	18	2 (11,1%)	13 (72,2%)	3 (16,6%)

Tabela 2 - Média dos ovos do Schistosoma mansoni *por grama de tecido hepático em camundongos com infecção única e com múltiplas infecções*

Grupos	nº	Média	Desvio padrão
Infecção simples	18	10.925,17	5.124,63
Infecções repetidas	26	12.139,03	5.343,64

Teste não paramétrico de Mann-Witney .

p > 0,05. Valor de p=0,6819

DISCUSSÃO

Os resultados observados indicaram claramente que os animais reinfectados pelo *S. mansoni* exibiram uma maior proporção de fibrose periportal em comparação com os animais com infecção única. Estes dados são válidos para o modelo murino. Não podem ser transpostos acriticamente para a patologia humana. Uma avaliação do modelo, considerado como representativo da fibrose *pipestem* descrita por Symmers[21] no homem, já foi apresentado[2]. Há nos dois casos semelhanças e diferenças, mas, no conjunto, o modelo murino aparece como o mais adequado, simples e barato, para estudos sobre a patogenia da forma grave da esquistossomose, como estudos recentes têm demonstrado[3 13 14]. A primeira e mais simples explicação para os resultados aqui obtidos basear-se-ia numa diferença de carga parasitária entre os dois grupos experimentais. Infecções múltiplas resultariam em acumulação de carga parasitária, um fator que já foi evidenciado como primordial para o desenvolvimento da forma hepato-esplênica[6 18]. Todavia, a contagem de ovos no fígado não revelou diferenças estatisticamente significantes entre os dois grupos. Os camundongos com infecção crônica pelo *S. mansoni* desenvolvem uma circulação colateral no sistema porto-cava, que pode desviar os vermes imaturos de uma segunda infecção para sítios extra-hepáticos periféricos, um habitat inadequado que causaria a morte de muitos vermes[17 23]. Talvez a destruição destes novos vermes e a morte de esquistossômulos, periodicamente, venham a estimular ou modificar o comportamento vascular intra-hepático ou outros fatores envolvidos na reatividade aos vermes e seus produtos. Fatores imunológicos podem ser particularmente estimulados, mas estes últimos não estão ainda bem definidos[8 10]. Fatores reacionais diversos apareceram em outro trabalho como a explicação mais plausível para a incapacidade de camundongos desnutridos desenvolverem o modelo da fibrose *pipestem*[13].

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Hepatic capillariasis in rats: a new model for testing antifibrotic drugs

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Abstract

Rats infected with the helminth *Capillaria hepatica* regularly develop septal hepatic fibrosis that may progress to cirrhosis in a relatively short time. Because of such characteristics, this experimental model was selected for testing drugs exhibiting antifibrosis potential, such as pentoxifylline, gadolinium chloride and vitamin A. Hepatic fibrosis was qualitatively and quantitatively evaluated in liver samples obtained by partial hepatectomy and at autopsy. The material was submitted to histological, biochemical and morphometric methods. A statistically significant reduction of fibrosis was obtained with pentoxifylline when administered intraperitoneally rather than intravenously. Gadolinium chloride showed moderate activity when administered prophylactically (before fibrosis had started), but showed a poor effect when fibrosis was well advanced. No modification of fibrosis was seen after vitamin A administration. Hydroxyproline content was correlated with morphometric measurements. The model appears to be adequate, since few animals die of the infection, fibrosis develops regularly in all animals, and the effects of different antifibrotic drugs and administration protocols can be easily detected.

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Key words
- *Capillaria hepatica*
- Hepatic fibrosis
- Septal fibrosis
- Antifibrotic drugs

Introduction

Several animal models of hepatic fibrosis have been utilized for testing drugs exhibiting antifibrotic potential. The models of hepatic fibrosis utilized are mainly those obtained in rats by repeated injections of carbon tetrachloride, by injections of porcine serum or following total main bile duct obstruction (1). Mice infected with *Schistosoma mansoni* have also been used (2). However, all of these models present limitations, especially due to individual variation in fibrosis development and high mortality, not counting the prolonged time involved and the high cost of some of them. Recently, it has been shown that rats chronically infected with the helminth *Capillaria hepatica* regularly develop progressive and diffuse septal hepatic fibrosis (3) similar to that seen in rats repeatedly injected with whole porcine serum (4,5). Fibrosis starts around the 40th day after infection, when the worms are already dead and the parasite-dependent, focal necrotic and inflammatory lesions are undergoing fibrous encapsulation and resorption.

Because septal fibrosis develops within a relatively short time (40-45 days) in 100% of the infected rats and shows a progressive course toward cirrhosis, this model has been considered to be one of the most adequate for testing antifibrotic drugs both in terms of their therapeutic and prophylactic effects.

This report concerns the response of hepatic fibrosis associated with *C. hepatica* infection to several drugs exhibiting antifibrotic potential. It was assumed that the results obtained would be worthwhile both for testing the model and the drugs.

Material and Methods

Wistar rats of both sexes weighing 170 to 300 g were infected with approximately 1,000 embryonated eggs of *C. hepatica* each, administered by gavage. The eggs were obtained from the livers of experimentally infected rats. The liver tissue was homogenized in a blender at 1,000 rpm, washed repeatedly in tap water, and left to sediment, until the supernatant fluid was completely clear. The sediment containing the immature eggs was placed on a Petri dish and covered with gauze humidified with 0.5% formalin. After 28-30 days at room temperature (26-28°C), the eggs became embryonated and were recovered and used to infect the animals. After inoculation the animals were divided at random into 5 experimental groups of five animals each as follows:

Group I. Infected control. The animals received two weekly subcutaneous (*sc*) injections of 0.85% saline.

Groups II and III. The animals were treated daily with 6 mg of pentoxifylline (Trental; Hoechst, São Paulo, SP, Brazil), administered intraperitoneally (*ip*) and intravenously (*iv*), respectively.

Group IV. The animals were treated with 1 ml of a 4 mM solution of gadolinium chloride (Sigma-66H3405; Sigma Chemical Co., St. Louis, MO, USA) administered *iv*.

Group V. The animals were treated with vitamin A (Arovit; Roche, São Paulo, SP, Brazil) twice a week by the *sc* route at the dose of 50,000 IU up to a total of 200,000 IU.

Treatment was started for all the groups on the 25th day after inoculation. To follow the course of treatment and to evaluate the preventive potential of the drugs, the animals were submitted to partial hepatectomy (surgical liver biopsy) one week after the end of treatment (45th day of infection). Treatment was resumed one week later, up to the time of sacrifice on the 95th day following infection. Table 1 summarizes the experimental protocols used.

For histological study, fragments of the liver collected during all the experimental phases were immediately fixed in Bouin's fixative for 6 h, preserved in 70% alcohol and routinely embedded in paraffin. Sections were stained with hematoxylin and eosin. In addition, the sirius red method for collagen was also used (6). The slides were blindly and independently evaluated by two pathologists

Table 1 - Schedule of treatment of fibrosis in rats with septal hepatic fibrosis associated with Capillaria hepatica infection.

*Time considered from the day of inoculation.

Group	Dose	Route	Duration of treatment (days)*	Hepatectomy*	Sacrifice*
I - Saline	1 ml	sc	25-45	45th and 65th	95th
II - Pentoxifylline	6 mg	ip	25-45	45th	95th
III - Pentoxifylline	6 mg	iv	25-45	45th	95th
IV - Gadolinium chloride	4 mM	iv	25-65	45th	95th
V - Vitamin A	50,000 IU	sc	25-39	44th	106th

by a semi-quantitative method, which considered fibrosis as absent (0), mild (+), moderate (++) and marked (+++) (see Figure 1). In case of disagreement, a consensus was reached before decoding the slides. Other portions of the liver were separated and submitted to the method of Bergman and Loxley (7) for measurement of hydroxyproline content.

Morphometric measurements were made on histological slides stained by the sirius red method and submitted to a computer image analyzing system (Leica Quantimet Q500MC, Cambridge, England). A total sectional area of 17.01 x 10^6 μm^2 per case was evaluated. A Leica Microstar IV microscope with a 4X objective was used to examine 9 microscopic fields at random. The sectional area of the fibrous tissue, red stained, was directly measured and calculated as percent of the total area examined.

An extra group was used to test the effect of gadolinium chloride on Kupffer cells. Normal rats were injected with a single dose or with 3 repeated doses of gadolinium chloride (4 mM/animal) one every 72 h. Twenty-four hours after the last injection, the animals received 1 ml of 10% India ink (colloidal carbon) *iv*.

Normal rats injected with India ink served as controls. The animals were sacrificed 24-48 h after injection of India ink and their livers submitted to histological examination by means of paraffin sections lightly stained with hematoxylin.

Results

Scattered focal lesions containing disintegrating parasites and their eggs varied in quantity but septal fibrosis was diffuse and appeared regularly in all infected untreated animals. By the 107th day of infection, the focal lesions had become small, encapsulated and frequently calcified, while septal fibrosis was more marked, being sometimes associated with areas of nodular regeneration of the hepatic parenchyma (Figure 1A).

Evaluation of the extent of septal fibrosis by a semi-quantitative histological method demonstrated that the groups treated *ip* with pentoxifylline exhibited a considerable decrease or even total absence of septal fibrosis (Figure 1B, C and D) as compared to the

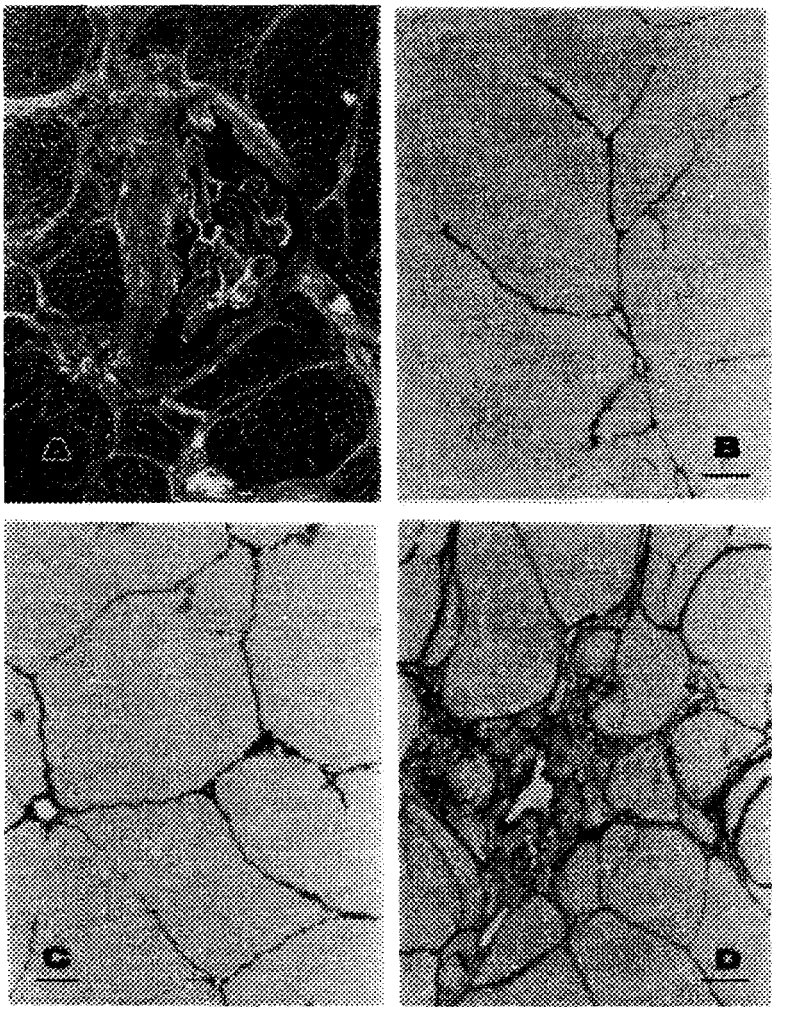

Figure 1 - A, Disintegrating adult worms (Capillaria hepatica) are seen at the center of the picture, surrounded by a necrotic-inflammatory reaction and a fibrous capsule. Septal fibrosis and regenerating hepatic nodules are also present. H & E, 120X (bar represents 100 μm). B, Representative of the mild (+) degree of septal fibrosis observed in rats treated with pentoxifylline by ip administration. Septa are few, thin and incomplete, and the majority terminate abruptly within the hepatic parenchyma. Sirius red staining. 100X (bar represents 100 μm). C and D, Representatives of the moderate (++) and marked (+++) degrees of septal fibrosis observed in infected rats treated with antifibrotic drugs. C, Septa circumscribe irregular areas of the hepatic parenchyma and show points of increased thickness (treated with gadolinium chloride); D, marked septal fibrosis delimiting hepatic nodules (cirrhosis) and forming areas of condensation, treated with vitamin A. Sirius red staining, 100X (bars represent 100 μm).

control group which presented a marked degree of fibrosis.

Morphometric evaluation confirmed the results obtained by semi-quantitative histology. The mean values for representative sectional areas of the liver revealed a considerable reduction of fibrosis for the groups treated with pentoxifylline (given *ip*), in comparison to untreated controls ($P<0.005$) (Table 2). The administration of vitamin A failed to modify the degree of liver fibrosis in a significant manner.

Hydroxyproline concentration (Table 3) also agreed with the data mentioned above but, in addition to indicating a statistically significant decrease in fibrosis for the group treated with pentoxifylline, it also showed similar results for gadolinium when administered before fibrosis had become established (prophylactic treatment).

The uptake of colloidal carbon by sinusoidal cells in normal rats treated with gadolinium chloride failed to show any difference when compared to untreated controls, with a similar number of sinusoidal cells containing carbon particles observed in both cases.

Data were analyzed by one-way ANOVA and the Student-Newman-Keuls test for group comparison.

Table 2 - Comparison of the mean values obtained by morphometric measurement of fibrosis in liver sections stained with sirius red from the various experimental groups.

*P<0.05 compared to control (Group I) (Student-Newman-Keuls' test). N = 5 for all groups.

Groups	Mean ± SD (μm^2)	Treatment
I	$19.28 \pm 2.17 \times 10^4$	PBS (0.85%)
II	$6.42 \pm 6.65 \times 10^{4*}$	Pentoxifylline (ip)
III	$16.94 \pm 6.76 \times 10^4$	Pentoxifylline (iv)
IV	$14.68 \pm 2.69 \times 10^4$	Gadolinium chloride (preventive)
V	$12.50 \pm 3.67 \times 10^4$	Vitamin A

Table 3 - Comparison of the mean values obtained by measurement of hepatic hydroxyproline content in the various experimental groups.

*P<0.05 compared to control (Group I) (Student-Newman-Keuls' test). N = 5 for all groups.

Groups	Mean ± SD (μm^2)	Treatment
I	8.84 ± 1.91	PBS (0.85%)
II	$5.69 \pm 0.49^*$	Pentoxifylline (ip)
III	7.85 ± 1.54	Pentoxifylline (iv)
IV	$6.41 \pm 0.89^*$	Gadolinium chloride (preventive)
V	7.08 ± 0.97	Vitamin A

Discussion

The model of septal fibrosis developed in rats infected with *C. hepatica* appeared to be adequate for testing antifibrotic drugs. Hepatic fibrosis was constant, uniform and progressive in infected controls and was significantly, albeit variably, affected by antifibrotic treatment. Since septal fibrosis does not appear before the 30th day of infection, drugs can be reliably tested with the present model for their prophylactic value when administered around the 25th day of infection or for their therapeutic action when administered after the 50th day.

Clear-cut positive results were obtained with pentoxifylline treatment, with the route of drug administration appearing to be crucial. The *ip* route yielded better results than the *iv* route. It is rather difficult to explain this finding, since the action of pentoxifylline seems to be quite complex. Pentoxifylline (methylxanthine) is known to inhibit collagen synthesis, suppress Kupffer cell activation and promote decrease of serum TNF-α (8,9). It can also inhibit collagen deposition and connective tissue cell proliferation (10). Probably, the plasma concentration and half-life of the drug differ according to the route of administration. Also, when injected *ip*, the drug would reach sinusoidal cells in the liver more rapidly or at more adequate concentrations than when systemically administered.

Sinusoidal cells play a pivotal role in septal fibrosis (11). As a matter of fact, the possibility of the drug interfering with sinusoidal cells stimulated us to use gadolinium chloride in this investigation. Gadolinium is

claimed to block phagocytosis by Kupffer cells (12-15).

In the present investigation, gadolinium initially yielded clear-cut antifibrotic results, but did not avoid the progress of fibrosis thereafter. The drug causes phenotypic alteration in Kupffer cells, causing them to produce excess TNF-α (14). Surprisingly, our results with the India ink test did not indicate that gadolinium altered the phagocytic capacity of Kupffer cells.

Vitamin A has yielded controversial antifibrotic results. High doses are considered to induce fibrosis in some studies (16), while others have found the opposite (17). High doses of vitamin A cause the Ito cells to be loaded with fat droplets. This may even depress the synthetic machinery of the cell through a space-occupying effect. Our results failed to show an antifibrotic effect when high doses of vitamin A were administered to rats with *C. hepatica*-associated septal fibrosis of the liver.

Further studies with different doses and protocols, with the present drugs or other compounds, should better characterize the antifibrotic properties of the agents used. The effects observed can also be compared in different models, since the antifibrotic activity may vary according to the pathogenesis of hepatic fibrosis. Hepatic fibrosis is usually associated with chronic inflammation and/or hepatocellular necrosis. Septal fibrosis of the liver induced in rats either by porcine serum (2,5) or by *C. hepatica* infection (3) is not preceded by outstanding necrosis or inflammation. Septal fibrosis seems to have an immunological basis (18), which turns the present model still more interesting. In conclusion, the results of the present investigation indicate that the model of hepatic septal fibrosis associated with *C. hepatica* infection of the rat is adequate for studies concerning the response of fibrosis to drug treatment and that it may also be valuable for investigations concerning the pathogenesis of liver fibrosis.

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Effect of Interferon-α on Experimental Septal Fibrosis of the Liver – Study with a New Model

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Interferon-α is used in antiviral therapy in humans, mainly for viral hepatitis B and C. An anti-fibrotic effect of interferon has been postulated even in the absence of anti-viral response, which suggests that interferon directly inhibits fibrogenesis. Rats infected with the helminth Capillaria hepatica *regularly develop diffuse septal fibrosis of the liver, which terminates in cirrhosis 40 days after inoculation.*

The aim of this study was to test the anti-fibrotic effect of interferon in this experimental model. Evaluation of fibrosis was made by three separate methods: semi-quantitative histology, computerized morphometry and hydroxyproline measurements. Treatment with interferon-α proved to inhibit the development of fibrosis in this model, especially when doses of 500,000 and 800,000 IU were used for 60 days. Besides confirming the anti-fibrotic potential of interferon-α on a non-viral new experimental model of hepatic fibrosis, a clear-cut dose-dependent effect was observed.

Key words: interferon-α - hepatic fibrosis - *Capillaria hepatica*

Patients with chronic hepatitis, especially due to hepatitis C, and presenting sustained responses to treatment with interferon-α, exhibited quantitative improvement of the degree of hepatic fibrosis (Manabe et al. 1993, Shiratori et al. 2000). The decrease in the amount of fibrosis was recorded even in non-responders to the anti-viral treatment and was seen to occur regardless of viral load, and the presence of inflammatory or degenerative changes (Guerret et al. 1999). Nevertheless, it remains rather difficult to determine from such clinical studies whether the effect on fibrosis was indeed direct or merely secondary to modulatory and anti-viral properties of the drug.

The anti-fibrotic effect of interferon-α on non-viral conditions has been tested with different experimental models of hepatic fibrosis. Positive results were observed with the classical model of carbon tetrachloride-induced hepatic fibrosis in rats (Pilette et al. 1997). However, the model of total bile duct ligation in rats has yielded both positive and negative results (Moreno & Muriel 1995). When these two models were actually compared, interferon was seen to inhibit fibrosis due to CCl4 administration, but not that derived from total bile duct ligation (Fort et al. 1998). As a contribution to this subject, the present paper deals with the effects of interferon-α on a new model of hepatic fibrosis in rats. As first described by Ferreira and Andrade (1993), rats infected with the helminth *Capillaria hepatica* regularly develop septal fibrosis of the liver. Septal fibrosis starts one month after infection and is already well developed around the 40th day, when the worms have already died and the focal direct parasite-dependent lesions exhibit encapsulation and signs of progressive resorption. Septal fibrosis is progressive from then on, and the late outcome is a full picture of cirrhosis. The histology is similar to that observed in rats repeatedly injected with whole pig serum, except for the presence of focal *C. hepatica* lesions (Paronetto & Popper 1966, Andrade 1991). Moreover, similar to the pig-serum model, septal fibrosis in capillariasis is not preceded by diffuse chronic inflammation or overt hepatocellular necrosis. Its pathogenesis has probably an immunological basis, as demonstrated for the pig-serum model (Bhunchet 1996). Antigenic materials sequestered within the focal parasitic lesions, being slowly released during prolonged periods of time, have been suspected as the main pathogenic factor in hepatic fibrosis associated with *C. hepatica* infection in rats.

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This new experimental model was used in the present study to test the effects of interferon-α on hepatic fibrosis.

MATERIALS AND METHODS

Adult Wistar rats of both sexes, weighing 170 to 300 g, were maintained on a commercial balanced diet and water *ad libitum*. They were infected with approximately 800 embryonated eggs of *C. hepatica*, administered by gavage. Twenty-five days after inoculation the animals were divided into four groups of five animals each, according to type and dose of treatment. Groups I, II and III were treated with subcutaneous injections of 0.5 ml of interferon-α in saline, administered daily in the doses of 100,000, 500,000 and 800,000 IU, respectively. The fourth group was used as control, treated under the same schedule, but with saline injections only. An hybrid, recombinant, non-species specific interferon-α, formed by an N-terminal segment composed by the aminoacids 1-60 HuIFN-αB and 61-166 HuIFN-αD, was used (Meister et al. 1986, Horisberger & Staritky 1987).

Treatment was administered from the 25th to the 65th day of infection. To follow the course of treatment a surgical liver biopsy was performed on the 45th day of infection. Sacrifice of all animals occurred on the 95th day after inoculation.

Samples of the liver obtained by biopsy and necropsy were submitted to histological, morphometrical and biochemical studies. Histology was made on sections of Bouin-fixed tissue, embedded in paraffin, which were stained with hematoxylin and eosin and picrosirius-red for collagen, according to Junqueira et al. (1979). The degree of fibrosis was first evaluated with picrosirius-red stained slides, by means of a semi-quantitative method, the sections being independently and blindly recorded by two observers. Fibrosis was arbitrarily recorded as (0) or absent (normal liver histology); mild (+) a few thin septa distributed in a few focal areas, usually around dead-worm lesions; moderate (++) when septal fibrosis was diffuse, but the septa were thin, usually incomplete, terminating within the liver parenchyma; and severe (+++) with numerous septa of variable thickness, circumscribing parenchymal nodules (cirrhosis). In case of disagreement, a settlement was reached by re-examining the sections before decoding them.

Morphometric measurements were made on histological sections stained with the picrosirius-red method, and analyzed by means of a Computerized Image Analyzing System (Leica Quantimet Q500MC, Cambridge, England). For such measurements a total sectional area of $17.01 \times 10^6 \mu m^2$ per case was evaluated. A 4x objective (Microscope Leica Microstar IV) was utilized in nine microscopic fields, randomly selected. The sectional area of the fibrous tissue, red stained, was directly measured and calculated as a percent of the total area examined.

Biochemical determination of hydroxyproline content was made according to the colorimetric method of Bergman and Loxley (1963).

Results were submitted to statistical analysis.

RESULTS

All infected control animals, that did not receive interferon, developed septal fibrosis of the liver, which was already well established by the 45th day of infection and that evolved toward a full picture of cirrhosis by the end of the experiment (Fig. 1). Fibrosis appeared as fine and long, thin, deeply sirius-red stained threads, formed along the acinar zone III and connecting central veins to central veins and also to portal spaces. In cases with marked degree of fibrosis the septa appeared thicker, exhibited condensation zones and presented numerous radiating branches that delimited nodular areas of parenchyma, connected central areas to portal spaces and sometimes ended abruptly within the hepatic parenchyma. The nodular zones of the hepatic parenchyma usually maintained its normal structure, with one-cell thick cords, but, later on, formed expansive regenerative nodules, with more than one-cell-thick cords (Fig. 2). Treatment with interferon considerably attenuated the degree of septal fibrosis. Although the fibrous septa still appeared circumscribing parenchymal nodules in some treated animals, these were focally distributed, usually in direct connection with focal dead-parasite lesions, and in none of them a complete morphological picture of cirrhosis was registered (Fig. 3).

The decrease in the amount of fibrosis appeared evident when the tissues were analyzed with semi-quantitative histological (Table I), morphometrical (Table II) and biochemical (Table III) methods. A dose-dependent effect was observed. With the dose of 100,000 IU the results were somewhat dubious. They did not reach statistical significance neither by measurement of hydroxyproline content, nor by the semi-quantitative histologic evaluation. However, morphometric measurements showed an anti-fibrotic effect, which was statistically significant. Results refer to necropsy material, since the group treated with the dose of 100,000 IU of interferon has not been subject to a previous biopsy.

Significant results appeared when the doses of 500,000 IU and especially 800,000 IU were applied. These high doses apparently did not cause any ill effect for the animals. They maintained good appearance of the body hair, were active, did

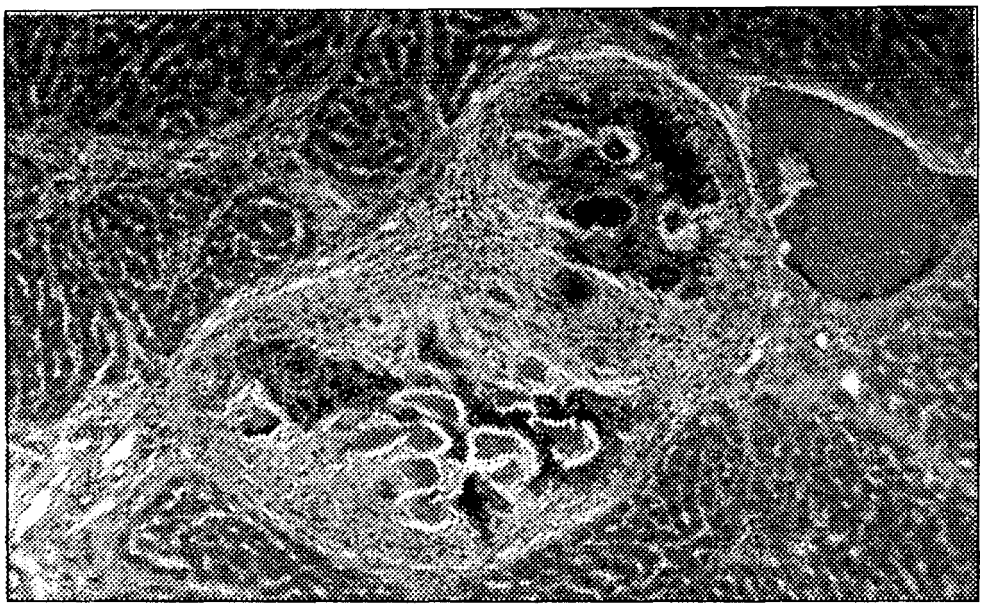

Fig. 1: focal lesion caused by *Capillaria hepatica* in the rat liver. Necrotic remnants of dead worms and eggs appear surrounded by an encapsulating fibrous reaction. The hepatic parenchyma is already dissected by thin fibrous septa. Hematoxylin and Eosin, 160X

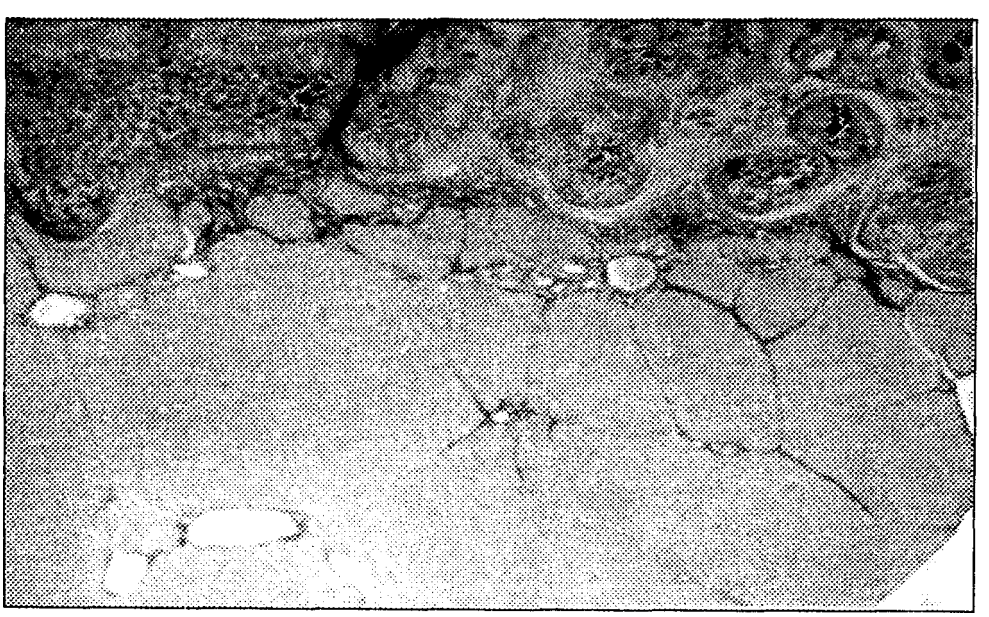

Fig. 2: numerous confluent parasite lesions caused by *Capillaria hepatica* in the rat liver are seen at the top of the picture, but septal fibrosis is minimal and only present at the immediate vicinity of the focal parasite lesion. The animal was treated with 800,000 IU of interferon-α for 60 days. Sirius-red stain for collagen, 25X

not loose weight or appetite, and did not differ in these aspects from the control rats receiving saline injections.

DISCUSSION

Septal fibrosis of the liver, associated with *C. hepatica* infection of rats, was clearly inhibited by interferon-α treatment. The anti-fibrogenic effect of the drug appeared dependent on the administration of high doses. As a matter of fact, the dose of 100,000 IU was first used in an attempt to reproduce the conditions in the paper by Moreno and Muriel (1995) in which fibrosis occurring in the model of total bile duct ligation in rats was successfully treated. Results were then slightly suggestive of an anti-fibrogenic effect, being statistically significant on

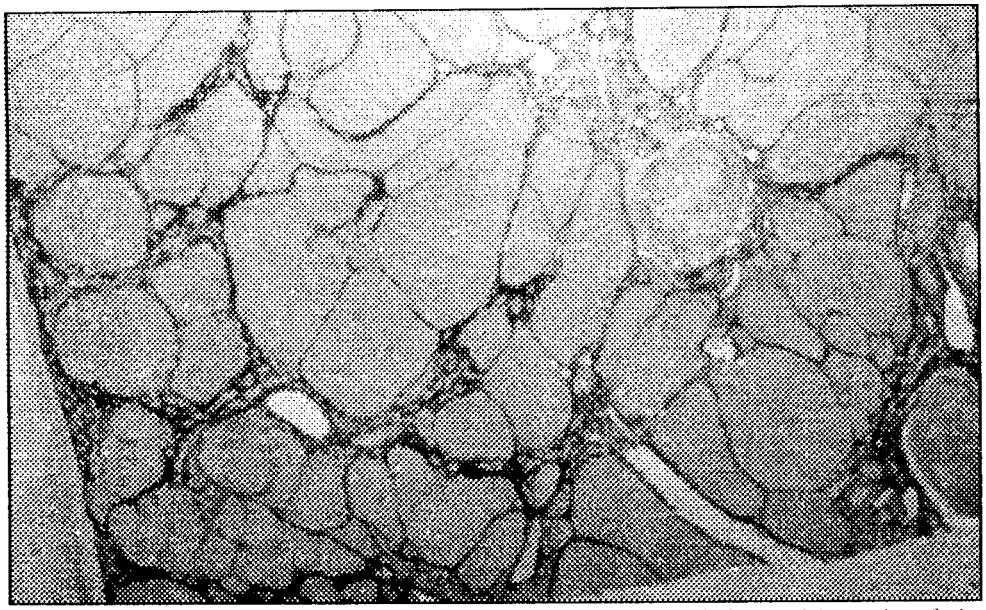

Fig. 3: control of interferon treatment. Septal fibrosis involves the entire liver and isolates nodular portion of the hepatic parenchyma, forming the picture of cirrhosis. A rounded residual fibrotic lesion, directly caused by *Capillaria hepatica*, is also present (arrows). Sirius-red stain for collagen, 25X

morphometric grounds only. Such discrepancy of results with different methods of fibrosis evaluation has also been observed in human studies when the anti-fibrotic effect was rather mild (Guerret et al. 1999). However, a clear-cut, dose-depend effect became evident when the animals were treated with 500,000 and 800,000 IU. The direct transposition

TABLE I

Semi-quantitative histological evaluation of fibrosis in rats with *Capillaria hepatica*-induced septal fibrosis of the liver after treatment with interferon-α (INF-α)

Groups	Biopsy	Autopsy	Treatment
Control (n=5)			Saline (PBS)
Group I			INF-α (100,000UI)
Animal 1	-		
Animal 2	-		
Animal 3	-		
Animal 4	-		
Animal 5	-		
Group II			INF-α (500,000 UI)
Animal 1			
Animal 2			
Animal 3			
Group III			INF-α (800,000 UI)
Animal 1			
Animal 2			
Animal 3			
Animal 4			
Animal 5			

TABLE II

Morphometric evaluation of hepatic fibrosis in rats with *Capillaria hepatica*-induced septal fibrosis of the liver

Groups	Average (μm^2)/SD	Treatment
Control (n=5)	$19.28x10^4 \pm 2.17x10^4$	PBS (0.85%)
I (n=5)	$13.41x10^4 \pm 5.5x10^4$	INF-α (100,000 UI)
II (n=3)	$10.31x10^4 \pm 3.59x10^4$	INF-α (500,000 UI)
III (n=5)	$5.69 x10^4 \pm 2.74x10^4$	INF-α (800,000 UI)

Differences between the control group and each one of the treated groups are highly significant ($P < 0.05$-Student-Newman-Keuls); INF-α: interferon-α

TABLE III

Hydroxiproline content measured in the livers of rats with *Capillaria hepatica*-induced septal fibrosis treated with interferon-α (INF-α)

Groups	Average µmol/g/SD	Treatment
Control 4 (n=5)	8.84 ± 1.92	PBS (0.85%)
I (n=5)	7.40 ± 1.27	INF-α (100,000 UI)
II (n=3)	4.13 ± 1.38	INF-α (500,000 UI)
III (n=5)	3.95 ± 0.73	INF-α (800,000 UI)

Differences between control (Group IV) and groups II and III are highly significant ($P < 0.05$, Student-Newman-Keuls)

of these doses for human use is certainly not adequate, but present demonstration is still of interest. Human beings can tolerate doses of interferon-α higher than 5 million units, although some adverse effects may become frequent. Effective doses and toxicities of drugs usually show large variations in different animal species. Demonstration of a dose-dependent effect of interferon on hepatic fibrosis may be important. Not only duration of treatment, but also the doses, are important factors observed for the anti-fibrotic effect of interferon-α in humans (Poynard et al. 1995, Degos et al. 1998). The side effects of interferons are not trivial, but effective interventions to reduce these effects may improve the quality of life of many patients (Cotler & Jensen 1999).

A possible direct effect of interferon-α on *C. hepatica* can be discarded. This helminth causes a self-limited infection to rats, all adult worms dying within the liver around the 25th-40th day after infection. Later on the involuting focal parasitic lesions are scattered distributed throughout the liver, their number showing little variation from case to case during the present experiment. All control cases exhibited severe degree of fibrosis (cirrhosis) by the end of experiment, regardless possible variation in number of parasitic lesions. Therefore, parasite load did not seem an important factor to be considered in the present circumstances in order to evaluate the anti-fibrosis effects of interferon-α.

The expression of α-isotype of actin in Ito's cells was seen to be markedly depressed in patients with chronic hepatitis submitted to interferon-α treatment, as immunohistochemically detected (Guido et al. 1996). This effect would interfere with the phenotype transformation of Ito's cells into myofibroblasts, a crucial step during extracellular matrix formation in the liver (Geerts et al. 1999).

Studies in vitro have demonstrated that interferon-α can also have other anti-fibrotic effects, such as increasing the synthesis of collagenases and glycosaminoglycan in cultured dermal fibroblats (and in interfering with cell-multiplication and collagen synthesis of cultured Ito's cells (Mallat et al. 1995, Geerts et al. 1999).

If septal hepatic fibrosis associated with *C. hepatica* infection in rats also depends on an immunological basis, the well known modulatory effect of interferon may be playing a role in preventing or degrading fibrosis in the experimental model now being considered.

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