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TESE DE DOUTORADO

ESTUDO SOBRE A PATOGENIA DA FIBROSE HEPÁTICA PERIPORTAL NA ESQUISTOSSOMOSE DO CAMUNDONGO.

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Estudo sobre a patogenia da fibrose hepática periportal na esquistossomose do camundongo

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1. INTRODUÇÃO

1.1 A ESQUISTOSSOMOSE

A esquistossomose é uma doença causada pela infecção por helmintos trematódeos do gênero *Schistosoma,* sendo que as 3 espécies mais importantes são: o *S. mansoni*, o *S. haematobium* e o *S. japonicum*. Milhões de pessoas em países subtropicais são infectados pelo *S.mansoni*, a espécie mais comum entre seres humanos (CHITSULO et al., 2000). No mundo, o número de indivíduos infectados pelo *S. mansoni* alcança a faixa de 180 a 200 milhões (WHO, 1998). No Brasil, a doença ocorre em 19 estados, com aproximadamente 26 milhões de habitantes expostos ao risco de infecção e cerca de 2,5 milhões de pessoas realmente infectadas (FUNASA, 1999). O Nordeste brasileiro é a área mais importante de ocorrência dessa doença no Brasil. Embora a esquistossomose seja um grande problema de saúde pública em vários países, ainda não se sabe exatamente por que menos de 10 % da população de uma área endêmica desenvolve a forma grave da doença, enquanto que a maioria dos indivíduos, mesmo vivendo nas mesmas condições, desenvolve uma infecção leve, muitas vezes assintomática.

A doença é iniciada quando o indivíduo (hospedeiro definitivo) se infecta ao entra em contacto com águas infestadas por cercárias liberadas pelo caramujo infectado. No Brasil, a *Biomphalaria glabrata* é o hospedeiro intermediário mais importante. A cercária penetra na pele do hospedeiro definitivo de forma ativa, perde a cauda e se transforma em esquistossômulo, forma sob a qual o parasita é mais vulnerável ao sistema imune. Os esquistossômulos migram através da corrente sanguínea e/ou vasos linfáticos para os pulmões, onde permanecem até se transformarem em larvas. Posteriormente, essas larvas migram para o sistema porta, onde se diferenciam em machos e fêmeas e se acasalam. As fêmeas iniciam a postura dos ovos, que são disseminados para vários órgãos do hospedeiro, em especial, para o intestino e o fígado. Nos pré-

capilares do sistema porta os ovos apreendidos induzem uma reação inflamatória granulomatosa, que bloqueia a difusão de substâncias tóxicas eliminadas pelos miracídios (REIS & ANDRADE, 1987). Produtos da inflamação, incluindo moléculas responsáveis por danos celulares, estimulam a diferenciação de células de Ito em miofibroblastos que secretam proteínas da matriz extracelular no espaço de Disse (GRIMAUD & BOROJEVIC, 1977).

As manifestações clínicas na fase aguda variam dependendo da área endêmica, intensidade do parasitismo, da resposta imune do indivíduo à infecção e do tratamento prescrito. A fase aguda ocorre entre a sexta e a oitava semana após exposição cercariana, sendo uma síndrome toxêmica, caracterizada pelo aparecimento de sintomas inespecíficos, distúrbios respiratórios e eosinofilia (RABELLO et al., 1997; GELFAND et al., 1981; LAMBERTUCCI et al., 1993; LAMBERTUCCI et al., 1997). A produção de citocinas pró-inflamatórias parece ser responsável pelo surgimento desses sintomas na fase aguda da infecção (RIBEIRO DE JESUS et al., 2002).

A grande maioria dos indivíduos evolui, na fase crônica, para a forma benigna da doença, conhecida como forma assintomática ou hepatointestinal (HI), que pouco difere das parasitoses intestinais comuns. Geralmente esta forma está associada a desconforto gastrointestinal, perda de apetite e dispepsia associados á presença de vermes adultos do esquistosoma na vascularização mesentérica (SAVIOLI et al., 1997). Entretanto, um baixo percentual de indivíduos na área endêmica (5 a 10%) desenvolve a forma grave da infecção, conhecida como forma hepatoesplênica (HE) compensada ou descompensada.

A forma HE é caracterizada por um aumento considerável do baço e do fígado devido à fibrose periportal, central e periférica, que leva a vários graus de obstrução dos ramos intra-hepáticos da veia porta. O aumento do baço se deve à hiperplasia dos elementos do sistema retículo-endotelial da polpa vermelha e da congestão passiva determinada pela hipertensão porta

(PESSOA & MARTINS, 1986; REY, 1991). A forma mais grave da doença, HE descompensada, inicia-se com a formação da circulação colateral, formando-se para varizes no esôfago e estômago, hiperesplenismo e ascite, podendo eventualmente levar a óbito (ANDRADE & VAN MARCK, 1984). A hepato-esplenomegalia caracterizada por fibrose periportal ou "pipestem" é mais comum entre indivíduos do sexo masculino, com faixa etária entre 10 a 40 anos. Sua instalação se faz precocemente, sendo necessários 5 a 15 anos para sua manifestação clínica e 3 a 5 anos para a evolução até a forma HE bem estabelecida (PRATA & BINA, 1968).

Vários estudos têm sido realizados com objetivo de esclarecer os mecanismos pelos quais apenas um pequeno percentual de indivíduos numa área endêmica evolui para a forma grave da doença na fase crônica. Descobrir quais são e como atuam esses diferentes fatores na evolução da esquistossomose para as formas graves, ainda é um desafio a ser atingido.

1.2 PATOGÊNESE DA FORMA HE

A patogênese da forma HE ainda é obscura, pois parece ser multifatorial. Dados da literatura demonstram que a prevalência da forma HE varia de forma considerável entre diferentes áreas endêmicas (LEHMAN et al., 1976; ANDRADE, 1998). Embora já tenha sido sugerido que o maior risco de desenvolvimento da forma grave se correlacione com uma maior intensidade de infecção, comunidades bem estudadas em diferentes países, nas quais os níveis de exposição foram semelhantes, apresentaram diferenças marcantes na prevalência dessa forma da doença (FULFORD et al., 1991). Essas observações sugerem que outros fatores, tais como idade, estado nutricional, carga genética, taxa de re-infecções e intensidade ou padrão da resposta imune aos antígenos do ovo possam influenciar na progressão da forma clínica assintomática para as formas graves.

Já é bem conhecido que a carga parasitária tem um papel essencial para o surgimento da forma HE. Em estudo realizado por Cheever (1968), pacientes que desenvolveram a fibrose periportal com obstrução vascular apresentaram deposição de inúmeros ovos do *S. mansoni* ao longo da trajetória da veia porta. Estes dados indicaram uma relação entre carga parasitária e desenvolvimento da fibrose periportal, e estão de acordo com o fato da esquistossomose grave com fibrose periportal não ser observada em indivíduos com infecção esquistossomótica leve. Entretanto, nem todos os indivíduos com elevada carga parasitária desenvolvem a forma grave da doença. Coura e Conceição (1981), ao estudarem indivíduos com elevada carga parasitária (indivíduos em fase de eliminação de 500 a mais de 2.000 ovos por grama de fezes) verificaram que estes não evoluíram com um quadro de esquistossomose hepatoesplênica. Em conjunto, estes estudos indicam que uma elevada carga parasitária é importante, porém não é o único fator responsável pelo surgimento da forma grave nos indivíduos infectados.

Vários estudos têm chamado a atenção para a importância da re-infecção como elemento determinante no desenvolvimento da forma grave. Em 1966, Katz e Brener demonstraram evidências de que a extinção espontânea do foco parasitário, portanto ausência de re-infecções, podia ser seguida de reversão da forma HE. Esses achados, juntamente com os estudos desenvolvidos por Coura et al. (1974) e Coura (1975), chamaram a atenção para o papel das re-infecções na instalação das formas HE. Anos mais tarde, Conceição et al. (1991), ao estudarem indivíduos infectados que migraram de áreas endêmicas para outras regiões, observaram uma redução nos índices da forma HE em pacientes tratados ambulatorialmente, sem possibilidade de re-infecção. Bina (1995) também verificou redução nos índices de hepatomegalia e esplenomegalia em uma área endêmica na qual houve quebra na transmissão da esquistossomose pelo combate à *B. glabrata*. Experimentalmente, Santos et al. (2000) evidenciaram um maior percentual de camundongos com fibrose pipestem quando submetidos á re-infecção, quando

comparados a camundongos provenientes de infecção simples, demonstrando que esse fator pode ser reproduzido experimentalmente em laboratório.

Outro elemento considerado importante para o desenvolvimento da HE é o *background* genético dos indivíduos infectados, especialmente a raça e o fator HLA, embora os resultados ainda não sejam conclusivos (PRATA, 1992). Trabalhos desenvolvidos anteriormente por Cardoso (1953) e Prata & Schroeder (1967) demonstraram que indivíduos do grupo racial negro apresentam maior resistência ao desenvolvimento da forma HE. Esses dados foram corroborados por dados mais recentes de Tavares Neto e Prata (1988) e Tavares-Neto (1995), através da demonstração de que pacientes do grupo racial branco apresentam maior freqüência da forma HE, bem como menor taxa de reversão dessa forma. No entanto, Bina (1995) encontrou uma significativa redução da prevalência da forma HE, no período de interrupção da transmissão, nos indivíduos do grupo racial branco. Alguns estudos têm apontado para um aumento de linfócitos $CD3^{-}HLA\text{-}DR^{-}$ em pacientes com a forma HE (MARTINS-FILHO et al., 1998/ 1999), porém esses estudos não esclarecem a participação desta subpopulação no determinismo da forma HE. Em trabalho desenvolvido por Secor et al. (1996), foi observada uma correlação entre a forma HE e o alelo DQB1*0201, embora os autores tenham chamado a atenção para a necessidade de estudos complementares.

1.3 FORMA HE E RESPOSTA IMUNE

No campo da imunologia, vários estudos têm demonstrado que, na esquistossomose mansoni, diversos mecanismos imunológicos estão envolvidos na evolução e manutenção da forma HE. Muitos desses trabalhos baseiam-se na correlação entre a resposta imune e o estado clínico dos pacientes infectados, sugerindo a existência de aspectos imunológicos distintos entre pacientes com a forma HE e pacientes com a forma HI. A investigação da resposta imune em

seres humanos tem sido realizada utilizando-se análises da reatividade de células mononucleares do sangue periférico (PBMC) frente a antígenos e/ou mitógenos solúveis (VIANA et al., 1994; COLLEY et al., 1986; MONTESANO, 1990a; MALAQUIAS et al., 1997).

Na fase aguda da doença, a resposta in vitro de PBMC apresenta alta reatividade a antígenos ovulares solúveis (SEA) (GAZINELLI et al., 1985). Com a cronicidade da doença, essa reatividade ao SEA tende a diminuir, sendo interpretada como resultado da modulação da resposta imune, que por sua vez estaria induzindo a diminuição do tamanho do granuloma (imunomodulação). Essa imunomodulação foi descrita pela primeira vez por Andrade e Warren (1964) em fígados de camundongos e, mais tarde, em seres humanos (RASO & NEVES 1965; RASO et al., 1978).

A capacidade de desenvolver mecanismos imuno-regulatórios que participariam no controle da resposta imune está geralmente associada ao desenvolvimento da forma HI, enquanto que a sua ausência estaria associada ao estabelecimento da forma HE (ELNNER, et al., 1981; TWEARD et al., 1983; COLLEY et al., 1986). Essa "falha" da modulação para resposta ao SEA é apontada como um dos fatores responsáveis pela fibrose hepática na esquistossomose. Em estudos desenvolvidos por Colley et al. (1986), quase todos os pacientes com sintomatologia aguda apresentaram uma resposta elevada ao SEA no estágio inicial da infecção. A maioria desses indivíduos, ao evoluírem para a forma HI da doença crônica, apresentou uma resposta moderada, sendo que apenas um pequeno número de pacientes permaneceram bons respondedores. Um alto percentual de indivíduos identificados clinicamente como HE continuaram a expressar elevados níveis de resposta ao SEA.

Pacientes com várias formas clínicas também apresentam anticorpos anti-idiotípicos contra SEA distintos quanto à habilidade de estimular, in vitro, a proliferação de linfócitos T anti-idiotípicos regulatórios presentes em preparações de PBMC de pacientes (MONTESANO et al.,

1989). Essas diferenças idiotípicas também foram detectadas sorologicamente através da identificação de anticorpos anti-idiotípicos policlonais ou monoclonais (MONTESANO et al., 1989/1990a). Estes autores levantaram a hipótese de que a eficácia do mecanismo imunoregulatório, bem como a intensidade da infecção e a imunogenética do hospedeiro, contribuem para as manifestações clínicas da esquistossomose em humanos cronicamente infectados.

A avaliação da resposta imune pelo *S. mansoni* aponta para uma dicotomia Th1/Th2 (SHER et al., 1991; PEARCE et al., 1991; GRYZCH et al., 1991). A reposta Th2 está geralmente mais associada a uma reação granulomatosa intensa com grande concentração de células inflamatórias e maior deposição de componentes da matriz extracelular do que a resposta Th1 (CHEEVER et al., 1992).

O papel de células Th1/Th2 também já foi investigado em indivíduos que apresentavam diferentes formas clínicas da esquistossomose. Em estudos desenvolvidos por Contigli et al. (1999), foi demonstrado que clones derivados de pacientes infectados apresentaram um perfil Th2/Th0, enquanto que clones derivados de pacientes hepatoesplênicos apresentaram um perfil Th1/Th0. Por sua vez, em trabalho desenvolvido por Viana e colaboradores (1994), foi observada uma elevada produção de IFN-γ e intensa proliferação após estimulação por SEA de PBMC de pacientes provenientes da área endêmica, quando comparados aos pacientes com a forma HI. Araújo et al. (1994) demonstraram que a inibição da secreção de IFN-γ por PBMC oriundas de pacientes de uma área endêmica não foi dependente da carga parasitária. Outros estudos neste contexto apresentam uma certa divergência de resultados, apontando para uma associação entre forma HE e resposta Th1, com altos níveis de TNF-α e IFN-γ e baixos níveis de IL-5 (MWATHA et al., 1998) ou baixa resposta Th2 (WILLIAMS et al., 1994).

Apesar de haver divergências quanto à associação entre o desenvolvimento da froma HE e um perfil de resposta Th, o papel de algumas citocinas na patogênese da forma grave esquistossomótica é provável. Estudos in vitro têm demonstrado que o IFN-γ é uma citocina potencialmente antifibrogênica, atuando através da inibição da produção de matriz extracelular pelas células de Ito, do aumento da atividade das colagenases hepáticas, da estimulação da síntese de metaloproteinases e da redução da produção dos inibidores de metaloproteinases (TIMP). TGF-β, IL-1 e IL-4 são citocinas fibrogênicas responsáveis por estimular a diferenciação de células de Ito em miofibroblastos e exercer efeitos opostos ao IFN-γ na síntese da matriz extracelular e de TIMPs (DUNCAN & BERMAN, 1985; TAMAI et al., 1995).

Em estudo desenvolvido por Zwingerberger et al. (1991), foi observado um desequilíbrio na relação IL-4/IFN-γ em cultura de linfócitos de pacientes infectados. Neste estudo, os níveis de IL-4 foram elevados e o nível de IFN-γ estavam reduzidos. Os níveis de IL-4 obtidos após estimulação de PBMC foram correlacionados positivamente com a intensidade da infecção enquanto que a produção de IFN-γ teve uma correlação negativa.

A análise do papel de citocinas na fibrose hepática em seres humanos é difícil por várias razões, seja porque os estudos devem ser conduzidos em áreas onde os indivíduos estão sob as mesmas condições de exposição ao patógeno e condições similares de habitação ou porque as análises devem ser conduzidas mais freqüentemente em pacientes com a doença ativa do que em indivíduos no estágio tardio. Além disso, esses estudos devem avaliar também covariáveis não imunológicas que podem confundir as análises. Estudos imunológicos em indivíduos infectados podem ser realizados apenas através do estudo de culturas de leucócitos, enquanto que fatores locais também são importantes para o desenvolvimento da fibrose (FRIEDMAN, 1999). Neste sentido, a utilização de modelos experimentais é, portanto, de grande importância para o

esclarecimento da relação entre os diversos fatores responsáveis pelo desenvolvimento da forma HE da esquistossomose.

1.4 MODELOS EXPERIMENTAIS

Modelos experimentais têm sido utilizados para melhor compreensão da anatomopatologia, patofisiologia e imunopatologia da infecção humana, bem como da ação dos quimioterápicos.

A fibrose portal ou "pipestem" (Symmers, 1904), encontrada em pacientes HE, é caracterizada clinicamente pela hepato-esplenomegalia, hipertensão portal, presença de varizes esofágicas e graus variáveis de pancitopenia (hiperesplenismo), freqüentemente com ausência de sinais de falha hepatocelular. Este quadro clínico tem sido reproduzido experimentalmente em chimpanzés com infecções densas por *S. mansoni* (SADUN et al., 1970) ou *S. japonicum* (LICHTENBERG et al., 1971). Entretanto, diferentemente da patologia humana, ele não é proveniente de uma hipertensão portal, provavelmente devido à extensa rede de vasos colaterais oriundos do sistema portal nesses animais. Além disso, esse modelo não se adequa ao uso freqüente, por serem animais de grande porte e de difícil obtenção.

Trabalhos utilizando babuínos indicam que estes como modelo experimental são os representantes mais próximos para o estudo da esquistossomose humana, devido à sua maior semelhança anatômica, genética e imunológica com o homem. Estes primatas adquirem infecção natural e desenvolvem as patologias hepáticas e intestinais da esquistossomose, além de apresentarem tamanho moderado, facilitando sua manipulação, o que permite o monitoramento da doença em vários órgãos. Farah et al. (2000) relacionaram a fibrose periportal em babuínos à re-infecção e à produção de TGF-β e IL-4. Embora o modelo experimental do babuíno tenha

vários pontos positivos, a sua utilização é de pouca valia, devido ao custo elevado para aquisição e manutenção desses animais. Além disso, para maior utilização desse modelo, haveria a necessidade de desenvolvimento de insumos específicos e obtenção de serviços de medicina veterinária adequados para primatas (NYINDO & FARAH, 1999).

O modelo murino apresenta-se como o mais adequado para o estudo, por apresentar vários paralelos com a doença humana, uma vez que estes animais apresentam a fibrose periportal comparável à desenvolvida no homem, por serem facilmente manipuláveis e pela disponibilidade de insumos adequados para a realização dos experimentos. A infecção densa pelo *S. mansoni* em camundongos produz uma hipertensão portal relacionada ao tamanho e número de granulomas, fatores estes que provavelmente não são relevantes para o mecanismo da hipertensão portal humana (CHEEVER, 1965). No entanto, camundongos infectados também apresentam lesões porto-venosas obstrutivas semelhantes às lesões em humanos, que são aparentemente responsáveis pela hipertensão portal.

Esse modelo começou a ser utilizado para o estudo da forma HE quando Warren, em 1966, observou que camundongos infectados com um a dois pares de vermes do *S. mansoni*, mantidos por longo tempo (mais que 16 semanas), desenvolveram sistematicamente uma fibrose periportal com concentração de granulomas periovulares e fibrose ao longo do espaço porta. Embora as lesões tenham sido observadas apenas microscopicamente, sua semelhança com a fibrose periportal humana foi evidente. Neste modelo, a fibrose periportal é resultante de uma deposição densa maciça e continua dos ovos do parasita ao longo das veias periportais intra-hepáticas dilatadas, com interconexão de espaços porta, bem como de espaço-porta com veias centrais, inflamação portal, granulomas periovulares, obstruções vasculares e teleangectasia (ANDRADE, 1987).

Em indivíduos infectados, foi observado que os ovos do parasita não se concentram em grandes espaços portais até que a fibrose periportal tenha sido instalada (CHEEVER, 1969). Nos camundongos, essas lesões ocorrem apenas em 30 - 50% dos animais, mesmo quando camundongos "inbred" são utilizados (ANDRADE & CHEEVER, 1993), sugerindo que o tempo da infecção e a postura de ovos pelo parasita não são os únicos fatores, mas que a reatividade por parte de alguns hospedeiros também é necessária para o desenvolvimento dessa patologia.

Um estudo desenvolvido por Eloi-Santos et al. (1992) apontou para a maior mortalidade de fêmeas infectadas pelo *S. mansoni* em comparação com machos CBA/J e C57BL/6, sugerindo que as fêmeas apresentam elevada sensibilidade ao desenvolvimento de vermes adultos, o que culmina com um maior índice de mortalidade. Disto resultou um maior enfoque no estudo da esquistossomose em camundongos machos, pela sua maior resistência.

Henderson et al. (1993) observaram o desenvolvimento do quadro de fibrose periportal uniforme e esplenomegalia evidente em 19-24% dos camundongos CBA/J machos com infecção crônica. A síndrome desenvolvida por esses animais caracterizou-se por uma esplenomegalia intensa, ascite, atrofia tímica, anemia severa e caquexia. Segundo os autores, a fibrose periportal foi associada a uma "falha" na produção de anticorpos anti-idiotípicos, sendo esse elemento interpretado como um fator que interfere na produção da modulação de granulomas periovulares em camundongos cronicamente infectados. A "falha" na produção de anticorpos também foi encontrada em humanos com fibrose de Symmer's (MONTESANO et al., 1990a, b). Por outro lado, estudos realizados por Andrade et al. (1998) utilizando camundongos Swiss Webster esplenectomizados e não esplenectomizados, demonstraram não haver diferenças significativas entre os níveis de anticorpos anti-idiotípicos e participação do baço nos animais que apresentaram ou não a fibrose periportal. A conclusão deste estudo foi de que o papel do baço é importante, mas não crucial para o desenvolvimento da lesão.

Mecanismos imunológicos presentes no modelo murino também assemelham-se aos encontrados em seres humanos. O estudo do padrão de citocinas produzidas no modelo murino levararam ao entendimento dos fatores que levam à regulação da resposta granulomatosa e da fibrose (WAHL et al., 1997; CHEEVER et al., 1998). A regulação da fibrose é freqüentemente independente da regulação do tamanho do granuloma, uma vez que grandes granulomas nem sempre estão associados a grandes fibroses (OLDS et al., 1989, CHEEVER et al., 1994, PHILLIPS et al., 1996). A fibrose hepática em camundongos infectados pelo *S. mansoni* está associada a uma resposta Th2 e produção de IL-13, muito embora a fibrose algumas vezes ocorra ao redor de granulomas formados por uma resposta Th1 (CHEN & BOROS, 1999; HERNANDEZ et al., 1999; HOFFMANN et al., 2000; HESSE et al., 2000).

Devido à grande quantidade de estudos que visam esclarecer o mecanismo de desenvolvimento da forma HE humana utilizando o modelo murino, torna-se importante uma revisão deste modelo no que diz respeito à reprodução de alterações vasculares, que têm um grande papel na patologia humana e o padrão de reposta imune desenvolvida por estes animais, correlacionando-os aos diferentes quadros histopatológicos.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o papel de fatores imunológicos e de fatores morfológicos indicativos da dinâmica vascular e das alterações da matriz extracelular na patogenia da fibrose periportal (pipestem) esquistossomótica do camundongo.

2.2 MANUSCRITO I

Descrever o perfil de resposta imune celular e humoral na esquistossomose murina, utilizando animias singênicos BALB/c com quandro histopatológico de fibrose periportal ou de granulomas isolados.

2.2.1 Específicos I

Caracterizar, em animais BALB/c com quadros histopatológicos de fibrose periportal ou granulomas isolados, os seguintes parâmetros imunológicos:

- Padrão de resposta Th1/Th2 através da dosagem de citocinas IL-4, IL-5 e IFN-γ no sobrenadante de cultura de células esplénicas estimuladas com mitógeno Con A e antígeno (SEA);
- Índice de linfoproliferação de cultura de células esplênicas estimuladas com mitógeno Con A e antígeno (SEA);
- Níveis de anticorpos anti-SEA dos isotipos IgG1, IgG2a, IgG2b e IgG3 e de IgE total no soro de animais infectados.

- Correlacionar os achados imunológicos com a carga parasitária e grau de fibrose nos dois grupos estudados.

2.3 MANUSCRITO II

Caracterizar o padrão de alterações vasculares portais e as alterações do tecido conjuntivo, comparativamente, em fígados de camundongos com fibrose periportal (pipestem) e naqueles com granulomas isolados, tanto após infecção única, como em infecções múltiplas.

2.3.1 Específicos II

- Caracterizar as alterações vasculares e avaliar o seu papel no desenvolvimento da fibrose periportal.
- Comparar histologicamente os dois quadros histopatológicos desenvolvidos por animais infectados pelo *S. mansoni* submetidos à infecção simples e à re-infecção.
- Avaliar o papel da carga parasitária no desenvolvimento dos dois quadros anatômicos básicos, na infecção simples e nas infecções repetidas.

3. MANUSCRITO I

The Mouse Model of Schistosomal Periportal (Pipestem) Fibrosis

I – Immunological Features.

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Abstract

Pathological studies with *Schistosoma mansoni*-infected BALB/c mice have shown that animals with chronic infection tend to present with one of two different hepatic pathological syndromes: periportal fibrosis with accumulation of granulomas (pipestem fibrosis-PF) or isolated, scattered periovular granulomas (IG) within the liver. In this study, we investigated a correlation between the development of histopathological syndromes and immune response pattern in a model of *S. mansoni* infection in BALB/c mice. Spleen cells from mice with PF and IG were stimulated with parasite egg antigen (SEA) or mitogen (Con A) at 20 and 40 weeks after *S. mansoni* infection. Cell proliferation and IFN-γ production were suppressed, especially 40 weeks after infection. Mice with PF had higher collagen content than mice with IG, although the number of eggs was similar in both groups studied. Both groups exhibited a Th2 response, with production of IL-4 and IL-5, and elevated IgG1 and IgE levels. Although levels of IL-4 and IL-5 were higher in cultures of PF, differences in anti-SEA IgG1 and total IgE levels were not significant. In addition, no significant differences were observed in the serum levels of anti-SEA IgG2a, IgG2b and IgG3 when mice from PF and IG groups were compared. These results support previous findings in laboratory mice that schistosome infection leads to increased production of Th2 cytokines. However, no fundamental differences in immunological pattern were found between the animals with the two anatomical forms of schistosomal liver fibrosis. Future studies may look for differences in immunopathology of fibrosis development and/or differences in periportal microvascular dynamics.

Keywords: *Schistosoma mansoni*; immune response; experimental model; schistosomiais

Introduction

Schistosoma mansoni-induced morbidity and mortality is of major concern in tropical regions of the world. In most individuals, the infection is relatively benign. Patients with the asymptomatic or intestinal form of the disease may only experience occasional gastrointestinal discomfort. However, 10% of infected people in endemic areas may develop the severe, hepatosplenic form of disease, characterized by the presence of periportal fibrosis (Symmer's clay pipestem fibrosis), splenomegaly, portal hypertension, collateral circulation, esophageal varices, ascites and hematemeses (Nash et al.1982, Chen & Mott 1988).

The factors that cause the development of periportal fibrosis associated with hepatosplenic disease are poorly understood. Although high worm load is considered the major factor in pathogenesis (Cheever, 1968; Coura & Conceição, 1981), additional factors, such as host genetics and immunologic response profiles, probably play a role, as not all heavily infected people will develop hepatosplenic disease. Various studies have investigated a correlation between the development of hepatosplenic disease and cytokine production. Some previous studies have demonstrated an association between Th2 cytokine production and intensity of infection (Zwingerber et al 1991; Williams et al 1994; Araújo et al 1994; Viana et al, 1994). These studies have found that the intensity of *S. mansoni* infection in schistosomiasis patients was positively correlated with IL-4 levels and negatively correlated with IFN-γ production. However, in another study, the development of hepatosplenic disease was associated with Th1 cytokine responses (Mwatha et al, 1998).

In the murine model, the pathogenesis of pipestem fibrosis resembles that in humans (Warren 1966; Andrade 1987). Periportal fibrosis appears only in a certain percentage of chronically infected mice, even when inbred animals are used, indicating that factors other

than worm burden, time and genetic background are involved (Andradre & Cheever 1993). The role of immune responses in granuloma formation has been well studied in *S. mansoni*-infected mice. However, little is known about the immune responses in mice with pipestem fibrosis. Various studies have demonstrated the importance of cytokine regulation of fibrosis (Henri et al. 2002; Vaillant et al. 2001; Cheever et al. 1998), although only one study has demonstrated an association between the production of anti-idiotype antibodies and the immune response in CBA/J mice (Montesano, 1997). In the present work, we compared the immune response of inbred mice with two different histopathological patterns following *S. mansoni* infection.

Materials and Methods

Mice

Male, four week-old BALB/c mice were used for infection with *S. mansoni*. Swiss Webster mice were used to obtain schistosome eggs for antigen preparations. All mice were raised and maintained at the animal facilities of Gonçalo Moniz Research Center, and provided with rodent diet and water *ad libitum*. All mice were sacrificed and treated in accordance with the Oswaldo Cruz Foundation Commission for Experiments in Laboratory Animals.

S. mansoni infection

BALB/c mice were infected transcutaneously with 30 *S. mansoni* cercariae of the Feira de Santana strain (Andrade and Sadigursky, 1985). This strain has been maintained through successive passages in laboratory-raised *Bimphalaria glabrata.* Sixteen weeks after infection, a liver biopsy was performed in order to identify the mice developing periportal fibrosis or scattered granulomas. Groups of mice were bled for serum collection and sacrificed 20 or 40 weeks after infection. To evaluate the degree of infection, a liver fragment from each mouse was digested in 0.5% NaOH, for counting eggs, according to the methodology described by Cheever (1968).

SEA preparation

To obtain *S. mansoni* eggs, Swiss Webster mice were infected with 100 *S. mansoni* cercariae of the Feira de Santana strain. Mice were sacrificed seven to eight weeks after infection, and livers were homogenized in 1.7% saline solution. Homogenate was then

filtered and centrifuged at 1300 RPM at 4° C. After purification in a percoll gradient, eggs were homogenized using a Potter homogenizer and submitted to four cycles of freezing and thawing. The suspension was then centrifuged at 14000 RPM at 4° C. The aqueous fraction was then collected, sterilized in a gamma-ray irradiator and the protein concentration determined by a fluorescent protein assay (Stein et al., 1973).

Histopathological analysis and collagen determination

Liver fragments were fixed in Bouin's fluid. The material was embedded in paraffin and the sections stained with hematoxylin and eosin, and with the picrosirius-red method for collagen (Junqueira et al., 1979). Histological sections stained with picrosirius-red were analyzed by automatic morphometry using a color digital video camera attached to an Olympus AX-70 microscope. The images were analyzed using the Image Pro Program (Media Cybernetics, Carlsbad, CA). The percentage of fibrosis was evaluated in a total section area of 12 mm^2 per animal.

For determination of collagen concentration, a liver fragment of approximately 200 mg was fixed in neutral buffered formalin, and the concentration of hydroxyproline was measured using the technique described by Bergman and Loxley (1963). Briefly, the liver fragment was digested in 6N HCl for 18 hr at 110°C. After neutralization with NaOH, levels of hydroxyproline were determined in a colorimetric assay using Ehrlich's reagent. Preparations were read in a spectrophotometer at 558 nm.

Antibody detection

The levels of SEA-specific IgG1, IgG2a, IgG2b and IgG3 antibodies were determined by ELISA. Microtiter plates (Nunc Maxi Sorp) were sensitized overnight at 4° C with SEA at

a concentration of 3 μg/well in 0.1 M $NaHCO_3$ (pH 9.6). After a blocking step with 5% fetal bovine serum in PBS, plates were incubated for two hours at room temperature with sera from infected mice. Plates were incubated with biotin anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (PharMingen), followed by addition of streptavidin-peroxidase conjugate (Sigma). Total serum IgE was determined by a sandwich ELISA using purified anti-IgE antibody for capture and biotinylated anti-IgE antibody for detection (PharMingem), followed by streptavidin-peroxidase conjugate (Sigma). Reaction was developed using TMB (3,3', 5,5'-tetramethylbenzidine) peroxidase substrate solution (Kirkegaard and Perry). Plates were read at 450 nm in a microplate reader (Molecular Devices, Sunnyvale, CA).

Spleen cell culture

Spleen cell suspensions were prepared in RPMI medium (Life Technologies, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% FCS (Hyclone, Logan, UT), L-glutamine (2 mM), vitamins, sodium pyruvate (1 mM), Hepes (10 mM), $5x10^{-5}$ M of 2-mercaptoethanol, and gentamycin (50 μg/ml) (Sigma). For cytokine determination, spleen cells were cultured in 24 well plates and stimulated with 1 μg/ml of concanavalin A (Con A) (Sigma) or 5 μg/ml SEA. Cell-free supernatants were collected after 48 hours and stored at -20°C for cytokine analysis. To evaluate the proliferative response, splenocytes were plated in 96-well plates at $4x10^5$/well in 200 μl and triplicate wells were stimulated with Con A or SEA for 72 hours, as described in figure legends. After pulsing with 1 μCi of [*methyl*-^{3}H] thymidine (Amersham, Little Chalfont, England) for 14-18 hours, proliferation was assessed by measurement of ^{3}H thymidine uptake in a β-plate counter (Packard, Meriden, CT).

Measurement of cytokine production

Supernatants of splenocyte cultures were tested for IFN-γ, IL-4, and IL-5 contents by ELISA, using antibody pairs from PharMingen, following manufacturer's instructions. Reaction was developed using the 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Kinkergaard & Perry Laboratories, Gaithersburg, MD) and read at 450nm.

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

Development of pipestem fibrosis in S. mansoni-*infected BALB/c mice*

BALB/c mice infected with *S. mansoni* were submitted to liver biopsy 16 weeks after infection, in order to identify the histopathological lesions of each individual. The histopathological diagnosis of liver biopsies demonstrated that 37 (59%) had periportal fibrosis, 10 (16%) mice had scattered granulomas, and 16 (25%) had a mixed pathology with scattered granulomas and discrete areas of fibrosis in the portal space. All mice with mixed histopathological lesions were excluded from the study.

Parasite burden was evaluated by the number of schistosome eggs found per gram of liver at 20 and 40 weeks after infection. No significant differences were observed between the number of eggs in mice with pipestem fibrosis and with scattered granulomas, at either 20 or 40 weeks following infection (Figure 1).

Quantification of liver collagen in S. mansoni-*infected mice with different histopathological patterns*

Collagen in liver tissue was quantified using two different methods. First, the amount of collagen was estimated by measuring the hydroxyproline content in liver fragments from *S. mansoni*-infected mice. Although the levels of hydroxyproline in liver fragments of mice with pipestem fibrosis were higher than those of mice with scattered granulomas, the differences were not statistically significant (Figure 2). An additional analysis was done using morphometric evaluation of picrosirius-red-stained sections. Twenty weeks after infection, the areas staining positively for collagen were significantly larger in liver sections from mice with pipestem fibrosis than in those from mice with scattered granulomas (PF= 31.75±2.5 μm^2 compared to IG=13.25±3.77 μm^2; $P<0.001$).

Comparison of proliferative and cytokine responses

The proliferation indices of spleen cells from mice with scattered granulomas upon stimulation with Con A or SEA were slightly higher compared to those of mice with pipestem fibrosis (Figure 3A). However, the differences were not statistically significant in the two time points analyzed. The response to SEA stimulation at 40 weeks after infection was decreased in both groups compared to the response observed at 20 weeks after infection (Figure 3B).

At 20 weeks after infection, the production of IFN-γ by spleen cells stimulated with SEA was very low in both groups of mice (Figure 4A). The levels of IFN-γ upon stimulation with Con A were significantly higher in cultures of spleen cells from mice with pipestem fibrosis (Figure 4A). The levels of IL-4 in cultures of spleen cells from mice with pipestem fibrosis were higher than those of mice with scattered granulomas upon stimulation with Con A and SEA at 20 weeks after infection (Figures 4B). The levels of IL-5 were similar upon stimulation with Con A or SEA at 20 weeks after infection (Figure 4C). At 40 weeks after infection, cytokine levels were low (IL-4 and IL-5) or undetectable (IFN-γ) in splenocyte cultures stimulated with SEA in both groups of mice (not shown).

Antibody production in S. mansoni*-infected mice*

The levels of SEA-specific antibodies were determined at 20 and 40 weeks after infection. No significant differences were observed in the levels of IgG1, IgG2a, IgG2b and IgG3 SEA-specific antibodies when animals with scattered granulomas and pipestem fibrosis were compared at the two time points analyzed (Figure 5). IgG1 was the

predominant isotype at 20 and 40 weeks after infection. Additionally, the levels of total serum IgE were similar in both groups of mice (Figure 5).

Discussion

To analyze the factors involved in the development of severe hepatosplenic schistosomiasis in humans seems a difficult task because of the numerous variables present. Genetic polymorphisms, variations in size and number of exposures to infection, immune responses to several stimuli, are some of the variables. In this study we compared the immune response of *S. mansoni*-infected mice at the two histopathological poles: the severe one (pipestem fibrosis) or the benign (isolated granulomas). In our model, syngeneic mice were infected in the same conditions (number of cercariae and time of exposure), allowing us to investigate the association between immune responses and histopathological findings regardless other factors.

A high percentage (59%) of BALB/c mice developed pipestem fibrosis upon *S. mansoni* infection. Studies using other mouse strains, such as outbred Swiss Webster (Santos, 2000) or inbred CBA/J (Henderson 1993), reported a smaller percentage of mice developing pipestem fibrosis after *S. mansoni* infection (11% and 20%, respectively). The finding that a higher percentage of BALB/c mice develop pipestem fibrosis suggests that host genetic factors may play a role, either in being more permissive to the worms or in the predisposition for the development of more marked tissue reactions.

One of the factors known to affect the collagen deposition and development of periportal fibrosis is the parasite load. Heavy *S. mansoni*-infections were associated with severe hepatosplenic form in *S. mansoni*-infected individuals (Cheever, 1969). Although the mice used in our experiments were exposed to the same number of parasites, it was possible for the parasite load to differ slightly between animals, which may have influenced the development of the histopathological lesions. However, no significant differences in the number of eggs in livers of mice with scattered granulomas and with pipestem fibrosis were

observed at the time points studied. This indicates that the number of eggs is not a sole factor to determine the progression to pipestem fibrosis.

In this study, an association was observed between levels of collagen, as indicated by hydroxyproline content of liver tissue, and severity of disease. This association was supported by morphometry analysis. Cheever (1997) has demonstrated that the association between egg numbers and fibrosis is not linear in mice. Although heavy infections induced higher percentage of hepatic fibrosis, the percentage of fibrosis per egg numbers was lower (Cheever, 1968). These findings are in agreement with the lack of correlation between egg numbers and fibrosis observed in our study.

Cytokines are important regulators of immuno-inflammatory responses and play a major role in the regulation of fibrosis deposition and degradation (Vaillant et al. 2001). Several reports in the literature demonstrated alterations in T helper cytokine profiles during the formation of granulomatous reaction (Pearce et al., 1991, Grzych et al, 1991, Fallon et al, 1998). Egg deposition is a stimulus to Th2-associated cytokine production, and thus the immune responses of mice during the chronic infection are associated with this profile (Grzych et al, 1991). In our study, proliferative responses production of IFN-γ was highly suppressed, especially after 40 weeks of infection. Although spleen cells of mice with pipestem fibrosis produced levels of Th2 cytokines (IL-4 and IL-5) higher than spleen cells from mice with scattered granulomas, the cytokine and anti-SEA isotype profile were similar. Thus, the development of pipestem fibrosis did not correlate with significant alterations in immunological profile at a systemic level.

It is possible that differences in egg deposition affect the hepatic microvasculature of *S. mansoni*-infected mice, to a point as to determine progression to pipestem fibrosis or

not. In fact, in another study we have found alterations in the periportal microvasculature of mice with pipestem fibrosis, which were absent in mice with scattered granulomas (Silva et al. submitted). This random fibrosis-promoting pattern of egg deposition may locally affect the production of fibrosing cytokines such as TGF-β and TNF-α, a possibility that we are currently investigating.

Acknowledgements

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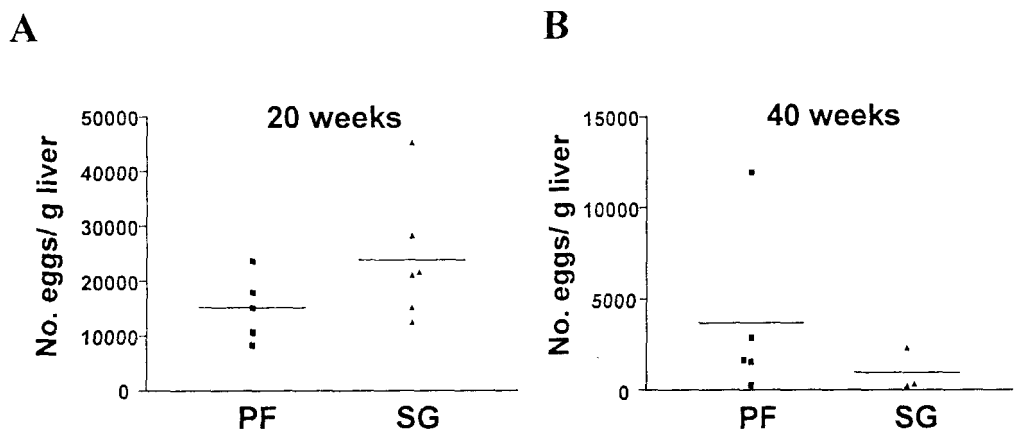

Figure 1. Parasite load in *S. mansoni*-infected mice. BALB/c mice were infected with 30 cercariae of *S. mansoni* and sacrificed 20 (A) or 40 (B) weeks after infection. The number of eggs was determined by counting after digestion of liver fragments. Data represent egg numbers per liver gram of individual mice. PF: pipestem fibrosis; SG: scattered granulomas.

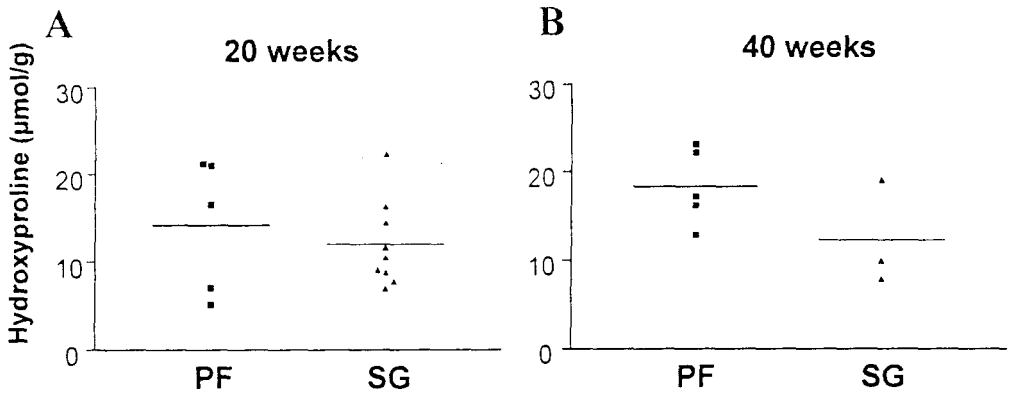

Figure 2: Quantification of liver collagen by hydroxyproline determination. Liver fragments of BALB/c mice were obtained 20 (A) or 40 (B) weeks after infection. The hydroxyproline levels per g of liver of individual mice were determined by colorimetric assay, as described in material and methods. PF: pipestem fibrosis; SG: scattered granulomas.

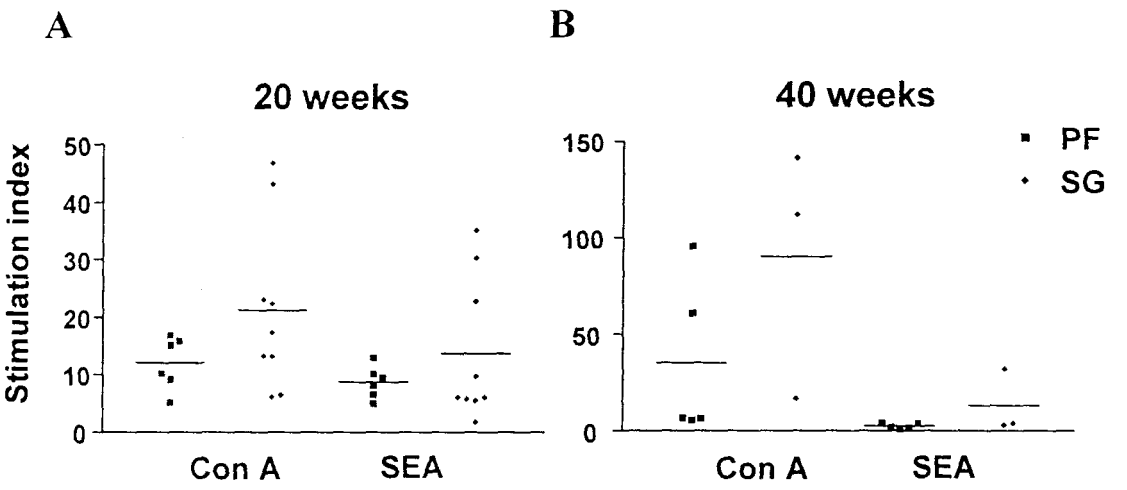

Figure 3: Proliferative responses of spleen cells from *S. mansoni*-infected mice. Spleen cells of BALB c mice with pipestem fibrosis or with scattered granulomas were obtained 20 (A) or 40 (B) weeks after infection and stimulated with Con A (1μg/ml) or SEA (5 μg/ml). Proliferative responses were determined 72 h later by ^{3}H-thymidine uptake. Data represent the stimulation index compared to untreated control cultures of individual mice. PF: pipestem fibrosis: SG: scattered granulomas.

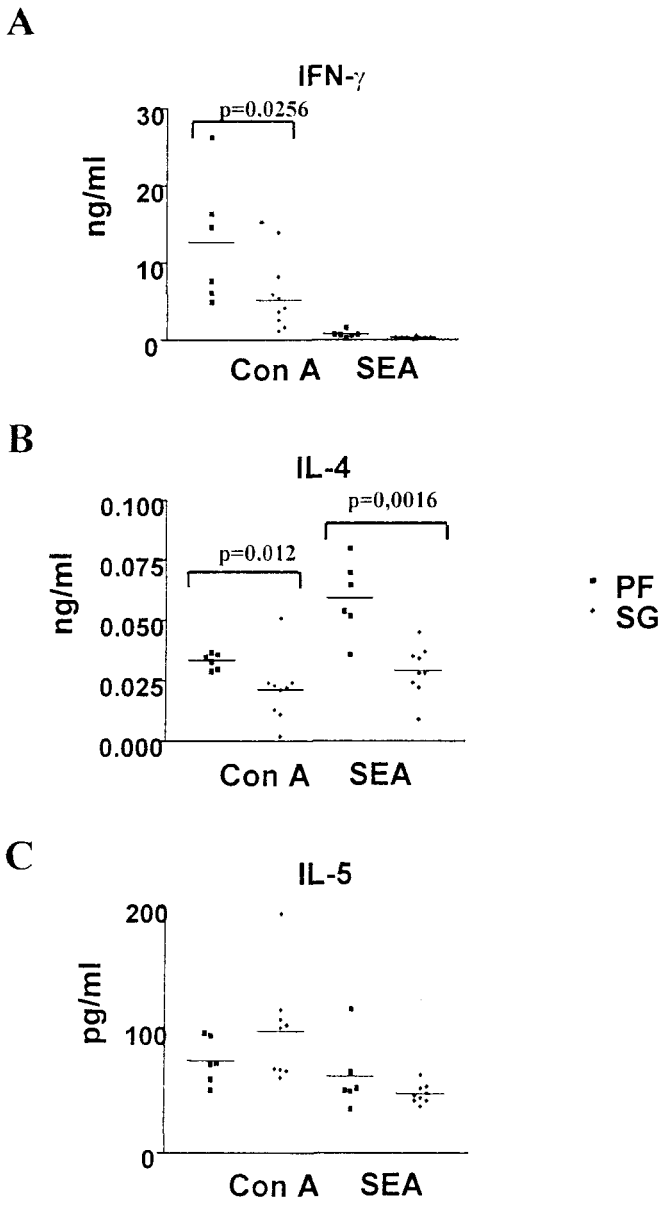

Figure 4: Cytokine production of spleen cells 20 weeks after *S. mansoni* infection. Spleen cells from BALB/c mice with pipestem fibrosis or with scattered granulomas were obtained 20 weeks after infection and cultured in 24-well plates in the presence of Con A (1μg/ml) or SEA (5 μg/ml). Cell-free supernatants were collected 48 h later and levels of IFN-γ (A), IL-4 (B) and IL-5 (C) were determined by ELISA. Data represent values of individual mice.

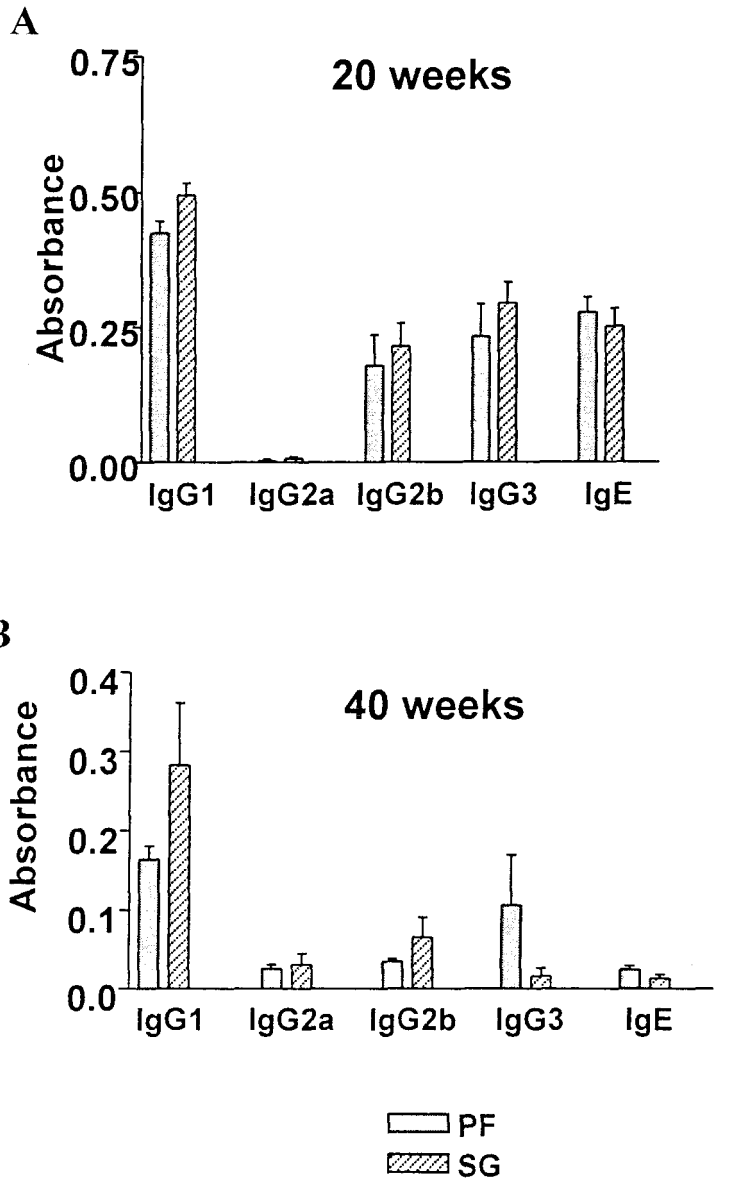

Figure 5: Analysis of antibody production in *S. mansoni*-infected mice. Serum levels of SEA-specific IgG1, IgG2a, IgG2b, IgG3 antibodies and total IgE antibodies were measured 20 (A) or 40 (B) weeks of infection by ELISA. Data represent the mean±SD of 6-9 (A) and 3-6 (B) mice per group.

4. MANUSCRITO II

The Mouse Model of Schistosomal Pipestem Fibrosis

II - Pathological Features

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Running title: Mouse model of pipestem fibrosis

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Abstract. The pathogenesis of schistosomal periportal fibrosis is still controversial. Mice chronically infected with *Schistosoma mansoni* develop either periportal (pipestem) fibrosis or scattered, isolated periovular granulomas in their livers. This study compared these two presentations using parasitological, biochemical and morphological techniques. We performed routine histology, automated morphometry, immunofluorescence and structural studies with plastic/corrosion casts of the portal vasculature. Mice subjected to single infections developed fibrosis intensely than those subjected to repeated cercarial exposure, with periportal fibrous tissue showing a prominent vascularization. From mice with periportal fibrosis, plastic casts revealed the presence of multiple collateral vessels sprouting from the main branches of the portal vein, severe amputation of the more delicate peripheral ramifications, and frequent distortions of the smallest branches. These vascular changes were absent from the livers of mice presenting the milder anatomical form. The evidence of vascular proliferation indicates that angiogenesis plays a role on the formation of granulation tissue, which precedes periportal fibrosis. Massive and continuous embolization of eggs to the portal circulation leads to obstruction and destruction of peripheral portal radicals and forces the opening of periportal collateral veins, thus facilitating the lodging of eggs at these locations. These changes may represent key factors in the pathogenesis of systematic periportal fibrosis of advanced-phase, chronic schistosomiasis.

Keywords: *Schistosoma mansoni*, Periportal (pipestem) fibrosis, Periovular granulomas, Hepatic fibrosis.

Introduction

The pathogenesis of schistosomal periportal (pipestem) fibrosis remains a controversial issue. It is recognized that the lesion described by Symmers in 1904[1] is highly characteristic, appears in heavily infected individuals, and represents the morphological substratum of hepatosplenic schistosomiasis. However, not all heavily infected individuals living in an endemic area will necessarily develop hepatosplenic disease. The influence of several factors, such as host age and gender, genetic background, nutrition, immunological status, and environmental factors have been discussed[2], but evidence for their specific roles is incomplete. There is no explanation for the finding that the majority of infected individuals present with isolated periovular granulomas in their livers, while only a few develop dense fibrous periportal plaques. Furthermore, it is not known why infected people who emigrate from an endemic area rarely develop pipestem fibrosis. If transmission is abolished from an endemic location, severe manifestations of schistosomisias, including hepatosplenic disease, tend to subside, which points to the influence of re-infection in pathogenesis[3]. Although there have been several thorough field studies and laboratory experiments performed to answer these questions,[4,5] the best hope for investigating which factors may influence pathogenesis requires the use of an experimental model.

A mouse model of pipestem fibrosis was described by Warren in 1966[6]. He noted that mice with relatively mild (1-2 pairs of worms) and prolonged (16 weeks) infections developed periportal fibrosis, characterized by a concentration of periovular granulomas in periportal spaces. Subsequently, Andrade[7] observed that portal vein plastic casts obtained from chronically infected mice showed severe amputation of small-sized veins at the liver periphery and the sprouting of dilated collaterals along the main extensions of the portal vein. He indicated that the deposition of eggs into the dilated portal vein collaterals allowed the periportal concentration of granulomas and fibrosis. Andrade & Cheever[8] showed that periportal fibrosis appeared only in a percentage of chronically infected mice, even when inbred mice were used. This indicated that other factors besides worm burden, duration of infection and genetic background are involved. In male CBA/J mice with chronic *S. mansoni* infection, Henderson et al.[9] observed that mice developing periportal fibrosis also exhibited massive splenomegaly and immunological abnormalities in the generation of modulatory anti-idiotypic antibodies. The presence of such antibody alterations was not confirmed by others, who demonstrated that schistosomal pipestem fibrosis could also develop in splenectomized mice.[10] A comparative immunological investigation of the two main pathological

presentations of hepatic schistosomiasis in mice did not reveal significant differences between them.[11] Intriguingly, pipestem fibrosis was not observed in undernourished mice.[12] Because the literature did not provide a clear explanation of factors involved in the pathogenesis of pipestem fibrosis, we decided to re-visit the mouse model of schistosomal pipestem fibrosis. This study is a comprehensive examination of hepatic pathology during re-infection, especially concerning vascular changes.

Materials and methods

Animals and Infection - BALB/C and Swiss Webster mice, male, weighing 15-20 g were each infected transcutaneously with approximately 30 *S. mansoni* cercariae of the Feira de Santana strain.[13] The cercariae were obtained from laboratory-raised and infected *Biomphalaria glabrata*. All the animals from each experiment were infected on the same day with the same preparation of cercariae. Half of the infected animals were submitted to repeated infection with 15 cercariae at each of five additional timepoints, at 15 day intervals, for a total of 6 infections. During the 16th week following the first infection, BALB/c mice were submitted to a surgical liver biopsy. Fragments of the liver were fixed in Bouin's fluid and embedded in paraffin for sectioning. Sections were stained with hematoxylin and eosin, for cellular identification, or picrosirius-red, for visualization of collagen.[14]

Experimental Groups - Based on the results of liver biopsy, the animals were segregated into 3 groups: 1) animals submitted to single infection with pipestem fibrosis (PF/SI); 2) animals submitted to single infection with scattered granulomas (SG/SI); and 3) animals submitted to re-infection with pipestem fibrosis (PF/RI).

Animals with an intermediate histologic picture, with features of both pipestem and isolated granulomas (mixed forms) were not included in subsequent analyses.

Sacrifice and Tissue Preparation - BALB/c infected-mice were killed between 20 and 24 weeks after the first cercarial exposure. Under ketamine/rompum anesthesia, the animals were exsanguinated by severing the brachial plexus. Fragments of liver were prepared as above, and stained with hematoxylin and eosin, picrosirius-red, or orcein for visualization of elastic fibers. Worms were recovered using the Duvall and De Witt[15] perfusion method. Egg isolation, quantification, and calculation of the number of eggs per gram of liver, were done

according to Cheever.[16] Collagen content was evaluated by measuring hydroxyproline content, according to method B of Bergman and Loxley.[17]

Immunofluorescence - Fragments of the liver were placed in small carton boxes filled with Tissue-Tek (Milles, Torrance, CA, USA) and immediately frozen in liquid nitrogen for 5 min. Blocks were then kept in air-tight plastic bottles at -70° C until sectioned in a cryostat at –20° C. The sections were collected and immediately washed in cold saline and treated with one of the following anti-sera: rabbit anti-human collagen I or rabbit anti-human laminin (kindly supplied by Dr Jean-Alexis Grimaud, Lyon, France). Primary antisera were diluted 1:20 or 1:50 in PBS. The secondary antibody was a fluoresceinated anti-rabbit IgG (Sigma, St. Louis, MO, USA), diluted 1:20 in a dilute solution of Evans blue. Control sections were incubated with saline only or with serum from an uninfected rat.

Automated morphometry - A total area of 12mm^2 on histologic sections was analyzed on per animal, for four animals from each group. All periovular granulomas with viable eggs were included. A spherical shape and normal size distribution were assumed. The percentage of fibrosis was calculated as the percentual area stained with picro-sirius-red examined. Similarly, the percentage of collagen or laminina was calculated as the percentage of the area that was fluorescently-labelled examined. For all automated morphometry, we used a color digital video camera attached to an Olympus AX-70 microscope with a 20x objective. The images were analyzed using the Image Pro Program (Media Cybernetics, Carlsbad, CA).

Vascular Injections - Infected Swiss Webster mice were biopsied at 16 weeks after infection and characterized as having pipestem fibrosis or isolated granulomas. Uninfected mice were used as controls. The animals were first intraperitoneally injected with 200µl of heparin. A 7% sodium citrate solution was fluxed through the portal system to clear blood from it. This was immediately followed by injection of 14% acetone solution of vinylite (vinyl acetate), under a manual-controlled low pressure, until uniform filling of the portal veins was achieved. After injection, the liver was removed and kept in water overnight at 4^{0}C. Then, the liver tissue was digested in a 50% hydrochloric acid solution. Forty-eight hours later the plastic cast was taken out of the acid, washed several times in water and alcohol, dried, and examined directly under a dissecting microscope. The plastic casts were analyzed using a SZX-9 Olympus stereoscopic microscope coupled to a DP-12 Olympus digital camera.

Statistical Analysis - Numbers of eggs, hydroxyproline content and morphometric data were analyzed using parametric or non-parametric methods. We used ANOVA to compare mean values for normally distributed data and Mann-Whitney (for two groups) or Wilcoxon (for three groups) tests to compare median values for non-normally distributed data.

Results

Generalities - The observed incidence of periportal pipestem fibrosis was approximately 60% for mice submitted to single infections and 55% for mice submitted to repeated infections (Table 1). The observed incidence of isolated granulomas was 18% in animals with single infection versus 23% among mice submitted to repeated infections. Figure 1 A-C shows the main histological criteria used for separating the two basic forms of hepatic schistosomiasis in mice. Picro-sirius stained liver sections from mice with periportal fibrosis had different appearance than those from mice with isolated granulomas when examined under low power (100x). Liver sections from mice with periportal fibrosis were characterized by a concentration of periovular granulomas in the periportal spaces, and variable amounts of intergranulomatous deposition of collagen forming the pipestem lesion (Figures 2A and B). No differences were observed in the hydroxyproline concentration, a measure of collagen content, when livers with pipestem lesions were compared to those with isolated periovular granulomas (Table 2). However, with a more sensitive method, automated morphometry, we found a larger picro-sirius stained area, positive for collagen, in livers with pipestem fibrosis compared to those with isolated granulomas (Figure 3).

Recovery of worms by portal vein perfusion at the 20th week following cercarial exposition revealed that mice submitted to re-infection that developed pipestem fibrosis (PF - RI) had a higher mean number of worms (21.0) than mice submitted to a single infection with pipestem fibrosis (PF-SI; mean = 9.0; p=0,02). Eggs were counted at two experimental timepoints: at 20 and 24 weeks after infection. We did not observe a difference in the number of eggs per gram of liver weight between pathologic groups (at 20 weeks post-infection than at 24 weeks (Table 3).

Vascular Changes - Besides the concentration of granulomas and fibrosis, which amplified the portal spaces in the cases of pipestem fibrosis, another striking difference from the

histologic pattern of isolated granulomas appeared in regard to vascular changes. While isolated periovular granulomas were avascular structures, with only an inconspicuous corona of blood vessels at their periphery, periportal fibrosis appeared as a richly vascularized fibrous tissue. Orcein-positive elastic fibers demarcated the wall of numerous vessels. Sometimes periovular granulomas had an intravascular location (Figure 1 D). Prominent vasculature was revealed when sections were marked for the presence of laminin and examined by immunofluorescence (Figure 2 C-F). Automated morphometric measurements indicated that the percentage of laminine content, a measure of the number of blood vessels, in pipestem fibrosis was much higher than in isolated granulomas (Figure 4). Furthermore, elevated laminine in granulomas appeared much more commonly in animals submitted to repeated infections.

Plastic casts obtained from the portal vasculature of uninfected control mice disclosed a vascular tree formed by regular dichotomous branching that progressively diminished in caliber down to fine, regular and delicate branches at the periphery (Figure 5 A and B). Casts obtained from the livers of mice presenting scattered isolated periovular granulomas were indistinguishable from those made from the livers of uninfected control mice. Some variation in the amount of fine vessels at the periphery of the cast was noted, more frequently in infected animals, but these could be attributed to technical variations. In contrast, striking changes appeared in the plastic casts from animals with pipestem fibrosis. There was considerable diminution or amputation of the more delicate peripheral ramifications, which gave a "dried-tree" appearance to the cast (Figure 5). In addition, the larger branches of the portal veins (1st, 2nd and 3rd orders), which appeared smooth in the uninfected control liver, presented numerous spikes, giving a "hairy" appearance to these branches (Figure 5 E). When observed under the dissecting microscope, the smallest vessels filled by the plastic revealed a series of abnormalities. These included sudden reduction of caliber (Figure 5 F and H), tortuosities, short-circuit (communications between neighboring vessels forming different geometric figures) (Figure 5 D), focal points of thinning and dilatation, sometimes alternating one with another, and formation of fine tufts, originating at terminal, partially-obliterated vessels (Figure 5 F).

As see in human patients, *S. mansoni* infection of mice can evolve to either one of the two polar forms of schistosomiasis: a mild or hepato-intestinal form, characterized by the presence of scattered periovular granulomas in the liver; and an advanced, severe, hepatosplenic form, with an almost pathognomonic hepatic form of periportal fibrosis. These aspects have been extensively studied in human material, [1,18,19] although they have received little attention in the mouse model of schistosomiasis.

A recent review on the mouse model of *S. mansoni* infection has called attention to the great research potential of this model.[20] One recent study of the pathogenesis of periportal (pipestem) fibrosis explored the potential role of the immunological Th-1 – Th2 dichotomy, but did not find fundamental differences between the two polar forms of schistosomiasis seen in infected mice.[11] This study pointed out that other lines of investigation, especially on the dynamics of connective tissue metabolism and its vascular supply, should be explored.

As a matter of fact, connective tissue metabolism and vascular supply are linked. In the present study, we observed a prominence of proliferating blood vessels in mice that displayed periportal fibrosis. The enlargement of the portal fibrous tissue in the mouse pipestem fibrosis is not only due to accumulation or aggregation of periovular granulomas, but to the formation of a new granulation tissue. This observation suggests that the arrival of schistosome eggs in periportal tissues leads to a complex inflammatory reaction. We hypothesize that factors involved in angiogenesis, such as VEGF, PDGF, and TGF-β, are involved. They have already been demonstrated in other experimental models of hepatic fibrosis.[21] The stimulation for the production of these fibrogenic factors, their receptors and inter-relationships may play a role in pathogenesis of schistosomal fibrosis and may explain individual variations of pathologic responses.

However, although cytokines and others factors may be involved, crucial features in the development of pipestem fibrosis are related to hepatic vascular changes induced by parasitological factors. Formation of peri-portal vascular cuffing was a prominent finding seen in human "pipestem" fibrosis when plastic casts were obtained with the injection/corrosion methodology.[22] It has been pointed out that the timing and sequence of the egg-induced intrahepatic vascular changes are crucial for these vascular changes to occur.[19] The sequential development of intrahepatic portal vein obstruction, followed by the

opening of periportal collateral veins and the continuous arrival of schistosome eggs into periportal vessels, appeared as essential steps in the pathogenesis of "pipestem" fibrosis.[19] Thus, for the development of this crucial vascular alteration, we believe that a series of sequential events occurs: a disseminated and sudden obstruction of portal branching veins by periovular granulomas, generation of intrahepatic portal hypertension, opening of periportal collaterals, and arrival of schistosome eggs into these new vascular canals. Of course, the production of numerous parasite eggs is essential. A proper (normal) immunological response is also important, as the data from undernourished mice indicated.[23] But the timing of the vascular changes and the arrival of new eggs seem essential for the end-result of "pipestem" fibrosis. Present results show that angiogenesis is a participant of schistosomal periportal fibrosis, and that vascular alterations of the portal vessels are numerous and varied in the mouse model, similar to those described in humans with schistosomal hepatosplenic disease.[22]

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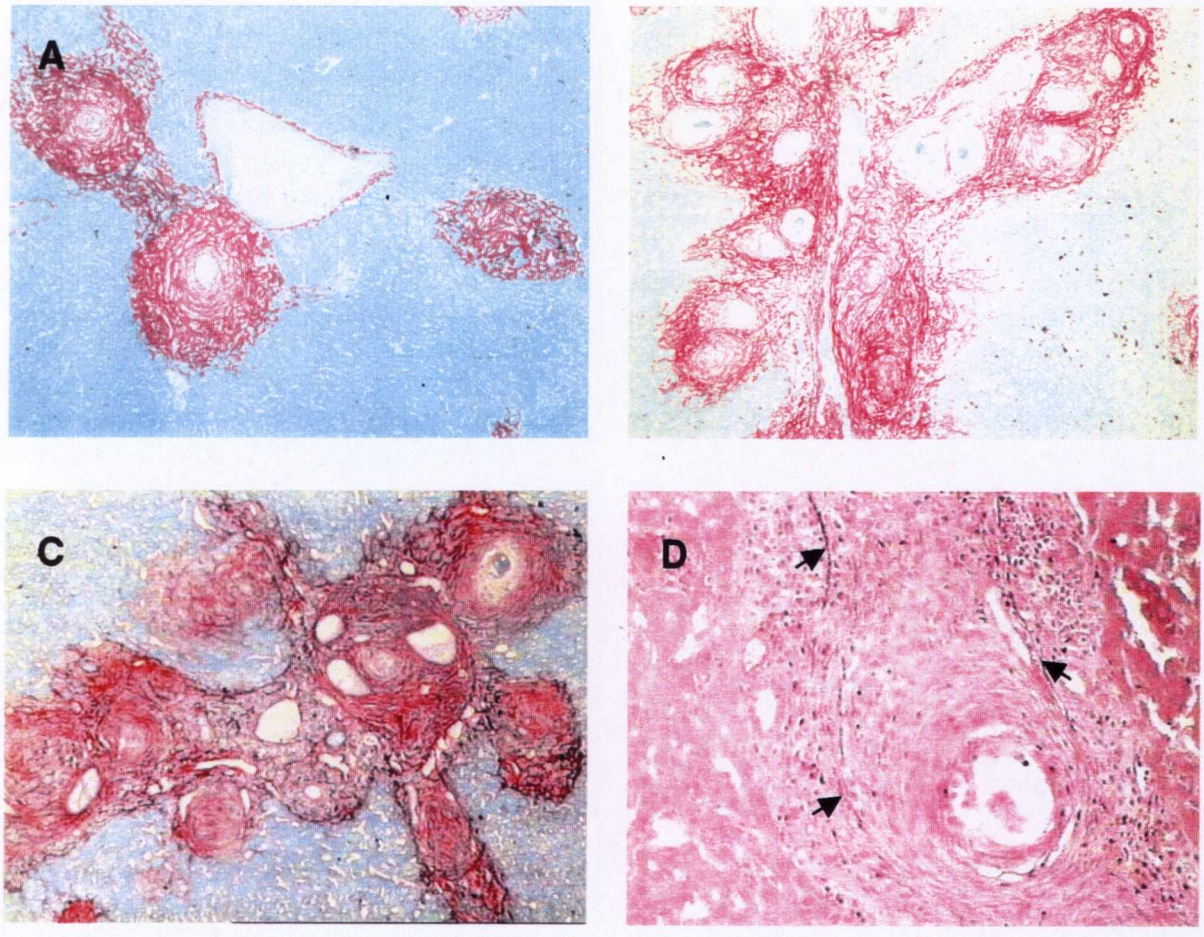

Figure 1. Liver Sections of *S. mansoni*-infected mice by optical microscopy. A - Isolated periovular granulomas in the mouse liver. Picro-sirius red, 100X; **B** and **C** – Schistosomal pipestem fibrosis. Portal spaces are enlarged, and connected one to the other, by extensive fibrosis. Picro-sirius red, 100X. **D** - Fine dark lines delineated the remains of a portal vessel, exhibiting a periovular granuloma in its interior. Case of pipestem fibrosis. Orcein stain for elastic tissue, 200X. Arrows indicate elastic fibers.

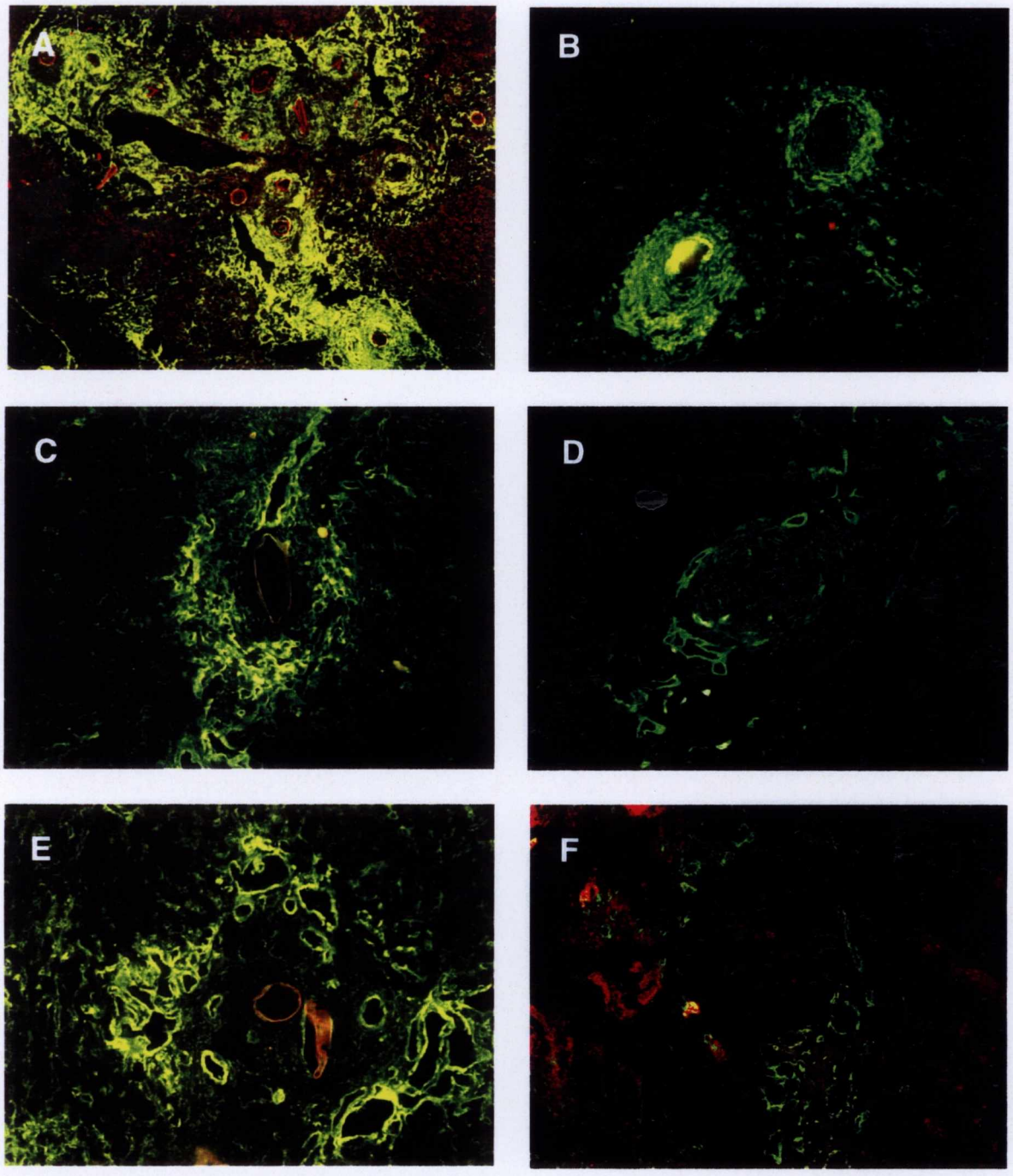

Figure 2. Confocal microscopy of indirect immunofluorescence showing aspects of schistosomal granulomatous lesions in the liver of BALB/c mice. A - Type I collagen in periportal (pipestem) fibrosis. **B** - Type I collagen in isolated periovular granulomas; **C** and **E** - Presence of numerous small laminin-positive blood vessels in sections of pipestem fibrosis; **C** = single infection; **E** = repeated infections; **D** and **F** - Idem, for isolated granulomas. Vessels present are much less numerous than in pipestem fibrosis. Objective Plan-Apo, 10X (**A**) and 20X (**B-F**).

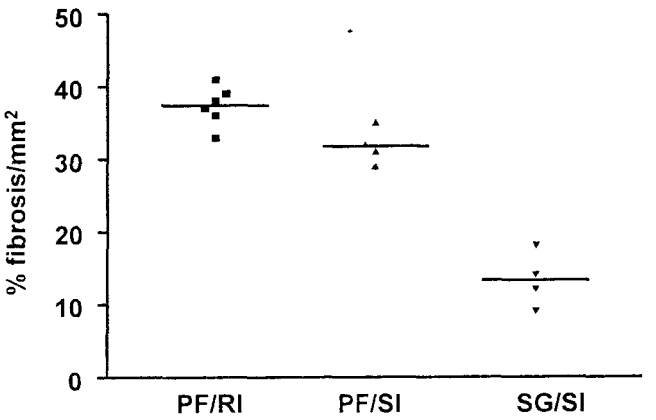

Figure 3. Evaluation of hepatic fibrous tissue by morphometry. Picro-sirius stained collagen in histological sections is expressed as a percentage of total fibrotic material examined in 12 mm^2 for each animal. BALB/c mice infected with *S. mansoni*, sacrificed 20 weeks after cercarial exposure. PS/RI: pipestem fibrosis from reinfection; PS/SI: pipestem fibrosis from single infection; IG/SI: isolated granulomas from single infection.

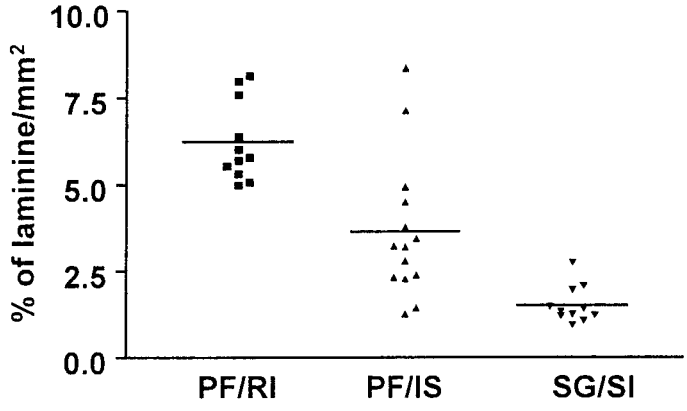

Figure 4. Percentual evaluation of vascular tissue, as measured by indirect immunofluorescence of laminin-positive vascular basement membrane. Cryostat sections from BALB/c mouse liver taken 24-weeks *S. mansoni* infection. PS/RI: pipestem fibrosis from reinfection; PS/SI: pipestem fibrosis from single infection; IG/SI; isolated granulomas from single infection.

Figure 5. Vynilite casts obtained from portal vasculature of mice infected with *Schistosoma mansoni*. **A** – A panoramic view of a portal vein cast from a normal mouse; **B** – A detailed view of a normal portal vein. **C** to **H** represent aspects of the portal vascular tree in cases of schistosomal hepatic pipestem fibrosis. **C** – A panoramic view showing decreased number of fine peripheral branches; sprouting of fine, short, periportal collaterals coming from the main branches and distortion. Details are depicted in **D**: short circuit (arrow); **E**: hairy appearance of collaterals. Arrows in **E** and **G** point to irregular branching, which also appear in **F** and **H** (sudden diminution of caliber). (page 69).

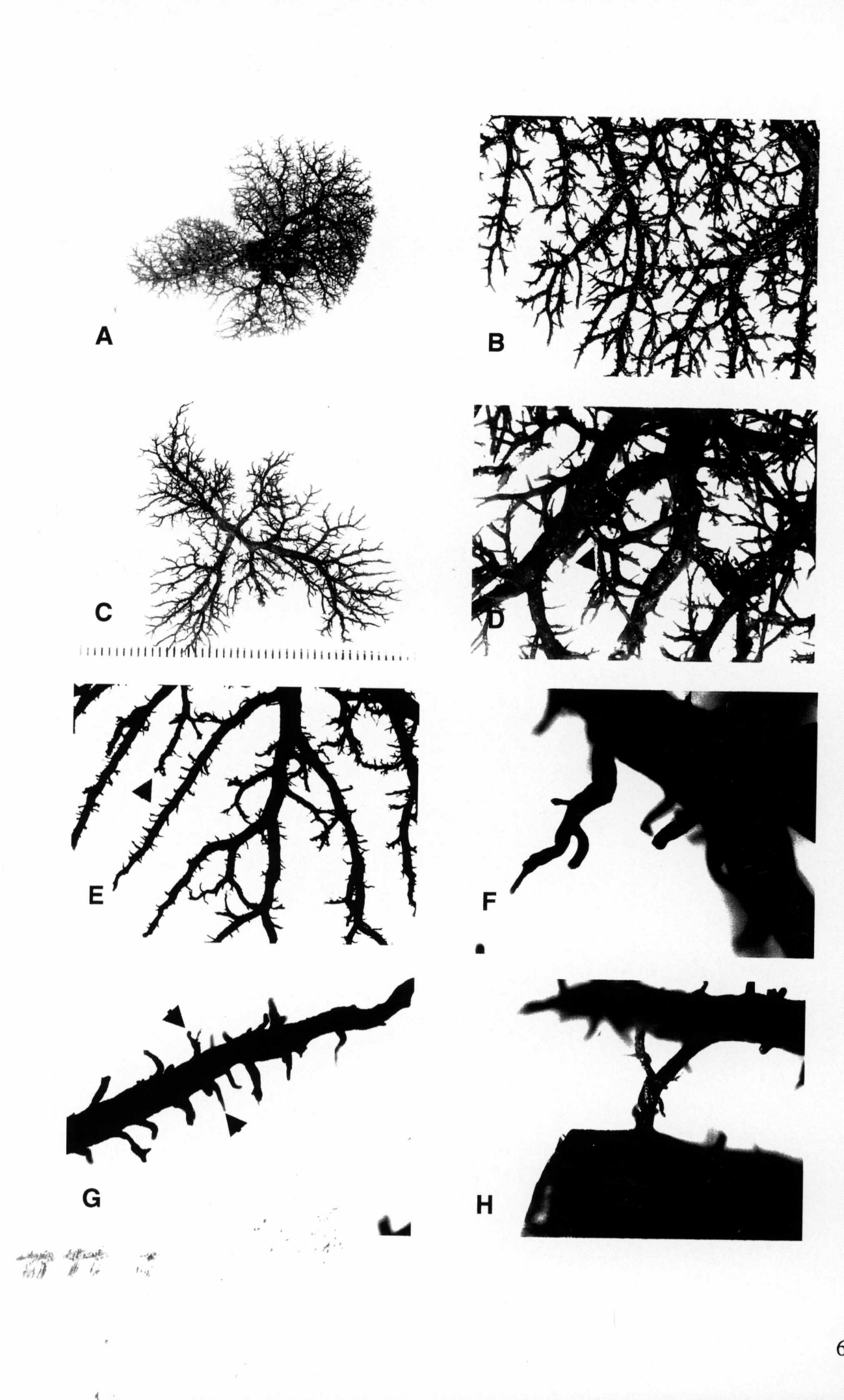
A
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Table 1. Classification of histopathological patterns as observed in biopsy material taken from mice chronically infected with *Schistosoma mansoni*.

Duration of infection	Type of infection	Number of animals	Pipestem fibrosis	Isolated granulomas	Indeterminate (Mixed)
20 weeks	Single	63	37 (59%)	10 (16%)	16 (25%)
	Repeated	60	36 (60%)	4 (7%)	20 (33%)
24 weeks	Single	30	20 (67%)	6 (20%)	4 (13%)
	Repeated	10	5 (50%)	4 (40%)	1 (10%)

Table 2. Quantification of liver collagen by hydroxyproline determination.

Duration of infection	Pipestem fibrosis Repeated Infections	Pipestem fibrosis Single Infection	Isolated granulomas. Single Infection.
	μmol hydroxiproline /gram liver		
20 weeks	9.25 ± 3.0	14.24 ± 7.64	12.0 ± 5.0
24 weeks	11.21 ± 1.65	13.07 ± 3.73	14.25 ± 3.45

Liver fragments of BALB/c mice were obtained 20 or 24 weeks after infection. Values represen the means ± SD of hydroxyproline levels per g of liver of 6 mice per group.

Table 3. **Estimates of the number of *Schistosoma mansoni* eggs per gram of liver tissue, according to type and duration of infection, in correlation with the anatomical lesions.**

Duration of infection	Pipestem fibrosis Repeated Infections	Pipestem fibrosis Single Infection	Isolated granulomas. Single Infection.
		Number of eggs/gram liver	
20 weeks	30.500 ± 9.177	17.380 ± 4.851	19.680 ± 6.127
24 weeks	10.450 ± 7.779	11.070 ± 10.100	4.795 ± 2.623

5. DISCUSSÃO

A esquistosomose mansoni vem sendo amplamente estudada no modelo experimental murino com o intuído de se identificar fatores genéticos, imunológicos e/ou patológicos que estariam determinando o desenvolvimento da forma HE em uma pequena parcela de animais infectados, semelhantemente ao que se encontra em seres humanos numa área endêmica. Em nosso trabalho, três fatores descritos na literatura como importantes para o desenvolvimento da fibrose periportal foram estudados: a participação de re-infecções sucessivas, a resposta imune do hospedeiro e alterações vasculares. Nossa meta inicial foi identificar fatores ou mecanismos importantes responsáveis pela patogenia da fibrose periportal e melhor caracterizar o modelo experimental murino para o entendimento da patologia humana. Em trabalho de revisão recentemente publicado, Cheever e colaboradores (2002) ressaltaram o grande potencial desse modelo e a sua pouca utilização no conhecimento da patogenia da fibrose periportal esquistossomótica.

Estudos da literatura apontam para a resposta imune como fator determinante do processo fibrogênico, correlacionando a maior produção de fibrose hepática com o perfil de produção de citocinas Th1/Th2 (CHEEVER et al. 1995; ASSEMAN et al. 1996). Já é sabido que, no início da infecção pelo *S. mansoni* (durante a penetração das cercárias até o completo desenvolvimento dos vermes), há um predomínio de resposta do tipo Th1, que é modificada posteriormente com a deposição de ovos pela fêmea para uma resposta Th2 (GRZYCH et al. 1991). Em nossos estudos, verificamos que o padrão Th2 foi predominante entre camundongos com quadros histopatológicos distintos, embora animais com quadro histopatológico de fibrose periportal tenham apresentado uma produção de IL-4 mais elevada do que a de animais com quadro de granulomas isolados. Com a cronicidade

da doença, a produção de citocinas e a resposta linfoproliferativa apresentaram-se suprimidas, embora com um padrão de resposta Th2. A produção de IL-4 por culturas de esplenócitos de camundongos tem sido associada à maior produção de fibrose hepática (DUNCAN & BERMAN, 1985; TAMAI et al. 1995), bem como à diminuição da produção de TNF-α, responsável pela caquexia e morte durante a fase aguda da doença (BRUNET et al. 1997, BRUNET et al. 1999). Nossos estudos concordam com os estudos em humanos que chamam a atenção para maior produção de citocinas do padrão Th2 em pacientes infectados numa área endêmica (WILLIAMS et al. 1994). Níveis de IFN-γ medidos no sobrenadante da cultura de esplenócitos foram baixos na 20ª semana e indetectáveis na 40ª semana pós-infecção. Estes dados também estão de acordo com o observado por Araújo e colaboradores (1994), em estudos com pacientes infectados em uma área endêmica.

Montesano e colaboradores (1997), utilizando camundongos CBA/J machos com diferentes quadros histopatológicos, correlacionaram a produção de anticorpos anti-idiotípicos com a produção de citocinas Th1 e conseqüente produção de granulomas menores, associada á forma branda da doença. O perfil predominante de resposta Th2 encontrado nos camundongos BALB/c e a maior porcentagem de animais com quadro de fibrose periportal em relação ao encontrado em camundongos CBA/J indica a importância do *background* genético na patogênese da doença. Camundongos BALB/c geralmente são mais susceptíveis a infecções, sendo amplamente utilizados em estudos sobre a patogenia da leishmaniose como padrão de resposta Th2, quando comparados a outras cepas de camundongos (SACKS & NOBEN-TRAUTH, 2002). Embora neste trabalho a produção de anticorpos anti-idiotípicos não tenha sido estudada, em trabalho publicado anteriormente pelo nosso grupo foi verificado que camundongos Swiss Webster submetidos a

esplenectomia antes e após a infecção pelo *S. mansoni*,desenvolveram fibrose periportal de forma idêntica a camundongos submetidos a uma falsa operação, sem qualquer correlação com a produção de anticorpos anti-idiotípicos (ANDRADE et al.,1998), reforçando mais uma vez que pode haver diferenças na resposta imune entre as diferentes linhagens de camundongos.

Nossos resultados sugerem, portanto, que o desenvolvimento da fibrose periportal não está correlacionado a alterações significativas no perfil de resposta imunológica a nível sistêmico. É possível que diferenças na deposição de ovos do *S. mansoni* interfira na microvascularização hepática, estimulando a produção local de citocinas fibrogênicas, tais como TGF-β e TNF-α em animais infectados, podendo até certo ponto determinar a progressão para maior deposição de tecido fibroso em torno da reação granulomatosa.

A partir dessa suposição, um segundo trabalho foi proposto com o objetivo de se estudar a dinâmica metabólica do tecido conjuntivo e seu suprimento vascular, uma vez que esses fatores estão interligados. No estudo desenvolvido por RAPPAPORT e colaboradores (1983), foi sugerido que o desenvolvimento da cirrose hepática estava invariavelmente acompanhada por uma intensa proliferação vascular. Esses autores sugeriram que o tecido de reparo e a fibrose poderiam representar uma rota para um fluxo da circulação colateral na cirrose. Essas observações foram confirmadas posteriormente, quando se revelou a presença de nódulos fibrosos regenerativos circundados por denso plexo vascular (YAMAMOTO et al., 1984; HARATAKE et al.1991).

Na presente investigação, detectou-se uma presença proeminente de vasos em proliferação nos animais com fibrose periportal. O alargamento dos espaços portais pela deposição de tecido fibroso, em animais com fibrose periportal, ocorreu não somente pela

acumulação e agregação de granulomas, mas também devido à formação de novos vasos, observada através da pesquisa de laminina em torno de reações granulomatosas. Ao determinarmos a sua localização e intensidade em torno da reação granulomatosa, através do uso do microscópio de imunofluorescência e avaliação morfométrica, foi possivel determinar indiretamente variações na proporção de vasos em torno de reações periovulares.

Camundongos BALB/c com quadro histopatológico de fibrose periportal apresentaram proliferação vascular mais evidente que camundongos com quadro histopatológico de granulomas isolados. O processo de re-infecção demonstrou maior influência sobre a proliferação de vasos, dentre os três grupos estudados, levando à suposição de que fatores imunológicos provenientes da constante exposição do hospedeiro ao antígeno do parasita estejam interferindo na resposta inflamatória local, promovendo maior proliferação de vasos. Essa suposição surgiu a partir da observação de que o número de ovos por grama de tecido hepático foi homogêneo entre os grupos estudados, embora o número de vermes adultos nos animais re-infectados tenha sido significativamente maior.

Fatores individuais podem estar interferindo sobre a microcirculação local, promovendo maior proliferação vascular, uma vez que animais com mesma carga genética e intensidade de infecção apresentaram quadros histopatológicos distintos. Essa observação chama a atenção para o fato de que a chegada de ovos do *S. mansoni* no tecido periportal pode estar estimulando uma complexa rede de reação inflamatória, gerando a produção de fatores angiogênicos, tais como VEGF, PDGF, TGF-β, dentre outros, que levariam à proliferação de vasos e deposição de tecido fibroso. O estímulo para a produção de fatores fibrogênicos, seus receptores e sua inter-relação pode estar exercendo um papel importante

na patogênese da fibrose esquistossomótica, podendo dessa forma explicar variações individuais.

Vermes adultos do *S. mansoni* têm como habitat natural o interior de vasos sanguíneos do sistema portal hepático e mesentérico. Os ovos depositados pelo parasita são carreados pela circulação sanguínea, causando obstrução de pequenos vasos no interior dos tecidos devido à formação da reação granulomatosa. Esse processo inflamatório que ocorre no interior da micro-circulação seria responsável pela baixa tensão de oxigênio no tecido adjacente, causando uma hipóxia temporária. Alguns estudos têm demonstrado a relação entre hipóxia e produção de VEGF (BROGI at al., 1996), bem como VPF como potentes mitógenos de células endoteliais (LEUNG et al., 1989; SENGER et al., 1983) e potentes mediadores de permeabilidade vascular e estimuladores de angiogênese (CONNOLLY et al., 1989; TAKESHITA et al., 1994). O VEGF é responsável pelo desenvolvimento da proliferação da microvasculatura associada à fibrogênese hepática, participando do remodelamento da arquitetura hepática na cirrose hepática (ROSMORDUC et al., 1999). Desta forma, o processo fibrogênico está relacionado a alterações vasculares hepáticas induzidas por fatores parasitários e da microcirculação do hospedeiro.

A formação de circulação colateral tem sido um achado proeminente em humanos com fibrose periportal, quando moldes plásticos foram obtidos da circulação hepática através do método de perfusão e digestão do tecido subjacente (BOGLIOLO, 1957; ANDRADE & CHEEVER, 1971). Tem sido sugerido que a formação de alterações vasculares ocorre em uma seqüência temporal induzida pela chegada de ovos na vascularização intra-hepática. O desenvolvimento de obstruções seqüenciais dos ramos da veia portal intra-hepática, seguida pela abertura de vasos colaterais e pela contínua chegada de ovos do esquistossoma parecem ser etapas essenciais na patogênese da fibrose periportal

(ANDRADE, 1987). Deste modo, o surgimento de alterações vasculares é um dos fatores necessários para uma série de eventos seqüenciais: disseminação e súbita obstrução vascular, geração de hipertensão portal, proliferação de vasos em torna da reação granulomatosa, abertura de colaterais na veia porta intra-hepática e chegada de ovos do parasita nos novos canais vasculares. Fatores envolvidos no desencadeamento de eventos são provavelmente multifatoriais. Naturalmente, a produção de inúmeros ovos pelo parasita é essencial, bem como a reatividade do hospedeiro aos ovos depositados. Em estudo realizado pelo nosso grupo, verificamos que animais desnutridos não desenvolveram quadro de fibrose periportal, o que pode ser o resultado da deficiência na produção de imunoglobulinas e elementos da matriz extracelular, como também de uma baixa fecundidade das fêmeas do *S. mansoni*, o que pode também alterar a deposição de ovos (OLIVEIRA et al., submetido para publicação). Deste modo, acreditamos que a chegada freqüente de ovos e alterações vasculares são essenciais para o desenvolvimento do quadro de fibrose periportal.

Nossos resultados confirmam e reforçam os dados acima analisados, que indicam ser o processo de angiogênese, juntamente com a produção de fatores fibrogênicos locais e alterações da circulação hepática, importantes para o desenvolvimento da fibrose periportal esquistossomótica. As alterações vasculares no sistema portal hepático são numerosas e variadas no modelo murino, semelhante ao descrito em humanos com a doença hepatoesplênica (ANDRADE & CHEEVER, 1971). Novos experimentos serão necessários para demonstrar a participação de citocinas angiogênicas e a cinética do processo fibroso no desenvolvimento dessa patologia.

6. CONCLUSÕES

1. Camundongos BALB/c apresentaram um elevado percentual de fibrose periportal mesmo quando submetidos a infecção simples.
2. O padrão de resposta Th2 foi dominante nos animais que desenvolveram tanto o quadro histopatológico de fibrose periportal, quanto o de granulomas isolados.
3. O modelo experimental do camundongo reproduz fielmente as alterações vasculares encontradas em seres humanos com fibrose periportal.
4. O processo de angiogênese estimulado pela reação granulomatosa no interior de vasos sanguíneos, está associado ao desenvolvimento da fibrose periportal no modelo murino.

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8. ANEXOS

Significance of Schistosomal Granuloma Modulation

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Hepatic Schistosoma mansoni *periovular granulomas undergo changes in size, cellular composition and appearance with time. This phenomenom, known as "immunological modulation", has been thought to reflect host immunological status. However, as modulation has not been observed outside the liver, participation of local factors, hitherto little considered, seems crucial. Components of the extracellular matrix of periovular granulomas of the mouse were particularly studied in three different organs (liver, lung and intestine) and during three periods of infection time (acute, intermediate and chronic) by means of histological, biochemical and imunofluorescence techniques, while quantitative data were evaluated by computerized morphometry, in order to investigate participation of local factors in granuloma modulation. Results confirmed modulation as a exclusively hepatic phenomenom, since pulmonary and intestinal granulomas, formed around mature eggs, did not change size and appearance with time. The matricial components which were investigated (Type I, III and IV collagens, fibronectin, laminin, proteoglycans and elastin) were found in all granulomas and in all organs examined. However, their presence was much more prominent in the liver. Elastin was only found in hepatic granulomas of chronic infection. The large amount of extracellular matrix components found in hepatic granulomas was the main change responsible for the morphological aspects of modulation. Therefore, the peculiar environment of the liver ultimately determines the changes identified in schistosomal granuloma as "modulation".*

Key words: modulation - granuloma - shistosomiasis - extracellular matrix

Hepatic granulomas formed around mature eggs of *Schistosoma mansoni* undergo changes in size, appearance and cellular constitution with time. This has been interpreted as a mechanism of host protection, with the host reactions becoming more economical and efficient during chronic infection (Andrade & Warren 1964). The immunological counterpart has been thoroughly investigated, both *in vivo* (Colley 1981, Chensue et al. 1993, Bogen et al. 1995) and *in vitro* (Doughty & Phillips 1982, Parra et al. 1991), including its cytokine patterns (Wynn & Cheever 1995, McKerrow 1997). However, there are data indicating that granulomas outside the liver may behave differently. Granulomas in the intestine were observed to be already modulated in the ileum from the very beginning, although modulation was said to occur in the colon and ileal Peyer's patch (Weinstock & Boros 1981, 1983). In the lungs granulomas presented the same morphology regardless the time of infection (Vidal et al. 1992, Eltoum et al. 1995). Such findings suggest that the so-called "immunological modulation" of periovular granulomas in schistosomiasis is mainly influenced by local factors. If this is so, the current interpretation of the immunological modulation concept needs to be revised.

To further investigate this possibility, a comparative study of size, appearance and matrix composition of granulomas formed in three different organs of mice (liver, intestine and lung), during three different periods of infection (early, intermediate and late), was undertaken. Methods of histopathology, immunofluorescence, biochemistry and morphometry were applied in an attempt to understand the real significance of the so-called "immunological modulation" of schistosomal granuloma.

MATERIALS AND METHODS

Thrity-one albino Swiss mice of both sexes, weighing 18-23 g, maintained with free access to a commercial balanced diet and water, were used. Animals were submitted to transcutaneous infection with 50 recently eliminated *S. mansoni* cercariae each. Later, animals were randomly divided into three groups, according to time of infection: first group (acute phase), animals sacrificed eight

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weeks following cercarial exposure; second group (intermediate phase), animals sacrificed at the 16th week after exposition; and third group (chronic phase), animals sacrificed at the 22nd weeks of infection.

To augment the chances of finding pulmonary granulomas, an extra group of mice was infected with 100 cercariae. Five animals were included in the acute phase group, and another five in the intermediate group. No animals infected with 100 cercariae survived to be included in the chronic group.

At the time of sacrifice the animals were anesthetized with ether, the abdomen was opened and the aorta severed. The liver was removed and weighed. The counting of worms was performed by examining the mesenteric veins and by smashing the liver between two slides. In this last case, several fragments to be submitted to other techniques were previously saved. A lobe of the lung was fixed by intra-tracheal injection of 10% formalin. Fragments of the liver, small intestine and lung were used for the following purposes:

Histology - Tissues were fixed in either pH 7.2 phosphate buffered 10% formalin in PBS or in Bouin's fluid. The 5μ-thick paraffin sections obtained were stained with the following methods: hematoxylin and eosin, sirius-red for collagen, Gomori' s silver impregnation for reticulum and Alcian-blue staining under two different pHs (2 and 7).

Counting of eggs - Fragments of the liver and intestines were weighted and digested overnight in 4% potassium hydroxide and the eggs counted according to Cheever's method (1970). Results were expressed as concentration of eggs per gram of tissue.

Immunofluorescence - Fragments of liver, intestines and lung were placed in small carton boxes filled with Tissue-Tek (Miles, USA) and immediately frozen in liquid nitrogen for 5 min. Blocks were then kept in air-thight plastic bottles at -80°C until sectioned in a criostat at -20°C. The sections were collected on clean glass slides and immediately washed in iced saline and treated with either one of the following anti-sera: anti-collagen I, anti-collagen III, anti-collagen IV, anti-fibronectin, anti-laminin, and anti-desmin. All anti-sera were kindly supplied by Dr Jean-Alexis Grimaud. The anti-sera were derived from human material and were prepared in rabbits. Their dilution at the moment of use varied from 1:50 and 1:100. Details about their preparation and specificity can be found elsewhere (Andrade & Grimaud 1986). The secondary antibody was a fluoresceinated anti-rabbit IgG (Sigma), diluted at 1:40 in a weak solution of Evans blue. Controls consisted of sections treated with saline only and sections treated with an irrelevant serum (normal rat serum).

Hydroxyproline measurements - Fragments of the liver were weighed and then submitted to biochemical determination of hydroxyproline content by the colorimetric method B of Bergman and Loxley (1963). Samples weighing from 100 to 250 mg were used. Hydroxyproline levels were corrected for intensity of infection by dividing total hepatic hydroxyproline (without correction for hydroxyproline levels in uninfected mice) by the number of *S. mansoni* eggs in the liver.

Morphometry - Histological sections either stained with hematoxylin and eosin or sirius-red were analyzed by automatic morphometry using a Leica Q500MC Image Processing and Analysis System (Leica Cambridge, Cambridge, England). For morphometric measurements a total sectional area of 17.01 $x10^6$ μm^2 per case was evaluated (Coutinho et al. 1997).

Statistical analysis - Numerical values obtained from egg counting, hydroxyproline measurement and morophometric data were analyzed using parametric tests. Analysis of variance with one or two factors (Anova) was used for identification of significance. Student-Newman-Keuls and the Student-t tests were applied for group differences. Results revealing $p<0.05$ were considered significant.

RESULTS

An average of five worm pairs per mouse was recovered, and that decreased to three during the intermediate and chronic phases of infection. When 100 cercariae were used, twice as many worms were counted. The number of eggs per gram of tissue was greater in the liver (14,713) than in the intestines (8,451), this difference being statistically significant ($p<0,05$). However, when morphometric measurements for numerical density were considered, the values for the intestine were greater than those for the liver (Table I). Morphometry also showed that the volume of the granulomas, considered as being spherical, decreased significantly in the liver, especially when those from the acute phase were compared with the chronic phase. No significant differences in granuloma volume were noted for those formed in the intestine or lung (Table II). Regarding volume density, which means the total volume occupied by the granulomas within a given unit of volume, the values for the liver were greater during the acute phase, decreasing thereafter. Such decrease did not occur for the intestine or lung. During the acute phase, granuloma volume density for the liver was greater than for the intestine and lung, but during the intermediate and chronic phases such difference lacked statistical significance (Table III).

TABLE I

Values for numerical density (Nn) of granulomas in different organs of mice infected with 50 *Schistosoma mansoni* cercariae during three periods of time, seven animals in each group

Organs	Time of infection (Nn x μm^3)		
	8 weeks	16 weeks	22 weeks
Lungs	1,36 ± 1,78	3,29 ± 4,35	5,38 ± 5,77
Liver	12,24 ± 2,09	14,89 ± 2,89	18,53 ± 4,29[a]
Intestines	15,59 ± 2,96	16,48 ± 2,42	21,93 ± 7,21[b]

a: P< 0,05 (Student-Newman Keuls) as compared to 8 weeks; *b*: p<0,05(Student-Newman Keuls) as compared to 8 and 16 weeks.

TABLE II

Average values for granuloma volume in different organs and at different periods of time in mice infected with 50 *Schistosoma mansoni* cercariae, seven animals in each group

Organs	Time of infection (x 10^4 μm^3)		
	8 weeks	16 weeks	22 weeks
Lungs	17,62 ± 27,62	44,28 ± 43,23	32,56 ± 39,19
Liver	481,53 ±161,51	252,98 ± 227,66	84,32 ± 26,22[a]
Intestines	22,96 ± 15,13	46,71 ± 17,13	16,96 ± 4,49

a: p<0,05(Student-Newman Keuls) as compared to 8 and 16 weeks.

TABLE III

Values for volume density (Vv) of periovular granulomas in different organs and at different periods of time in mice infected with 50 *Schistosoma mansoni* cercariae, seven animals in each group

Organs	Time of infection		
	8 weeks	16 weeks	22 weeks
Lungs	0,00 ± 0,00	0,01 ± 0,01	0,01 ± 0,02
Liver	0,18 ± 0,05	0,012 ± 0,05	0,07 ± 0,02[a]
Intestines	0,04 ± 0,02	0,09 ± 0,03a	0,04 ± 0,03

a: p<0.05(Student-Newman Keuls).

Histological evaluation was concerned only with periovular granulomas having a well preserved central miracidium. Old, involuting granulomas were not considered. Granulomas were abundant in sections from the liver. During the acute stage they appeared as predominantely exudative, frequently exhibiting a central halo of necrosis and a sprinkling of polymorphonuclear eosinophils (Fig. 1). Collagen fibers were thin and loosely arranged in a concentric disposition. Under immunofluorescence microscopy, Type III collagen appeared more abundant than type I, this latter being represented by fibers that seemed thicker and straight, rather than wavy, when compared with Type III collagen (Fig. 7A, B). Fibronectin formed a prominent network of fine fibrils all over the granuloma area (Fig. 7C). Type IV collagen and laminin were absent from periovular granulomas, revealing only basement membranes in small blood vessels situated at the granuloma periphery (Fig. 7D). A pale, diffuse Alcian blue staining marked all granulomas. This staining was similarly observed with either acidic and neutral pH. Within the structures of the miracidia, the Alcian blue staining became more accentuated, especially when a neutral pH was employed.

During the intermediate phase hepatic granulomas differed from those of the acute phase in showing a tendency to confluence and in being smaller and with the cells and collagen fibers becoming more packed. Eosinophils were less numerous while macrophages and fibroblasts became more prominent.

At the chronic stage periovular granulomas were especially characterized by being smaller, well delimited and more fibrotic than before (Fig. 2). Collagen fibers were thicker, concentrically distributed, and strongly fluorescent when tested

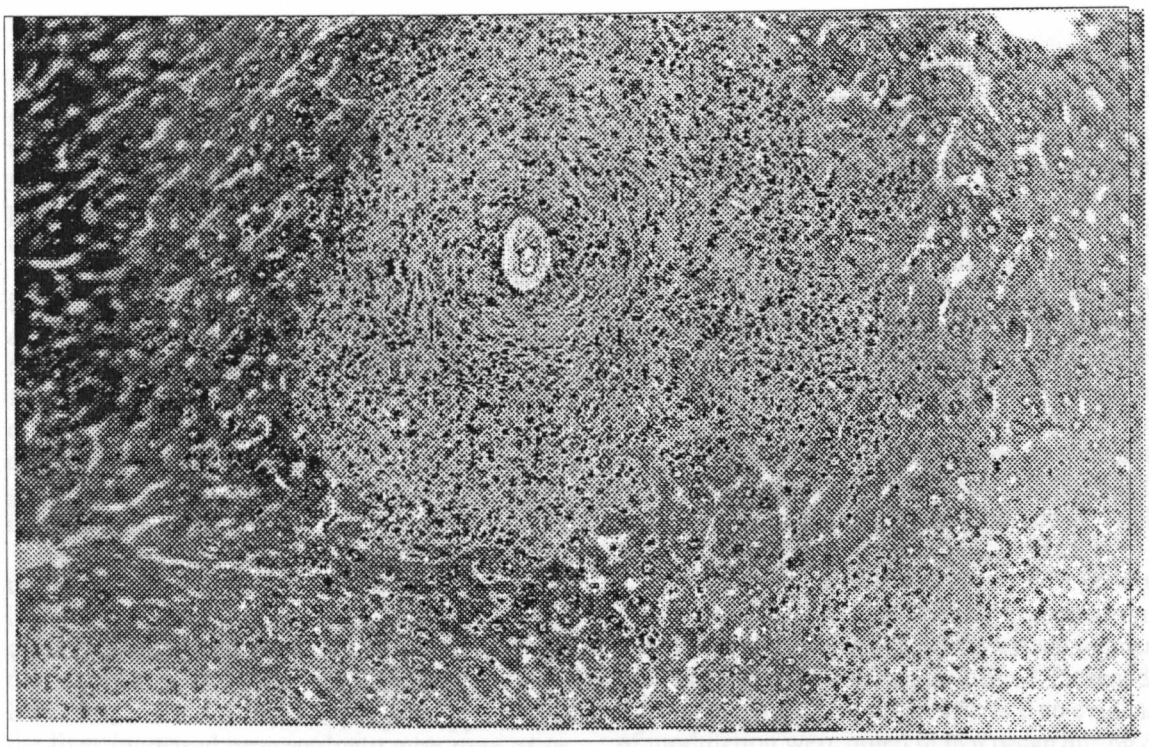

Fig 1: acute phase periovular granuloma in the liver. A large and irregular inflammatory area appears around a central schistosome egg. Central necrosis is present, although scanty. H & E, 64X.

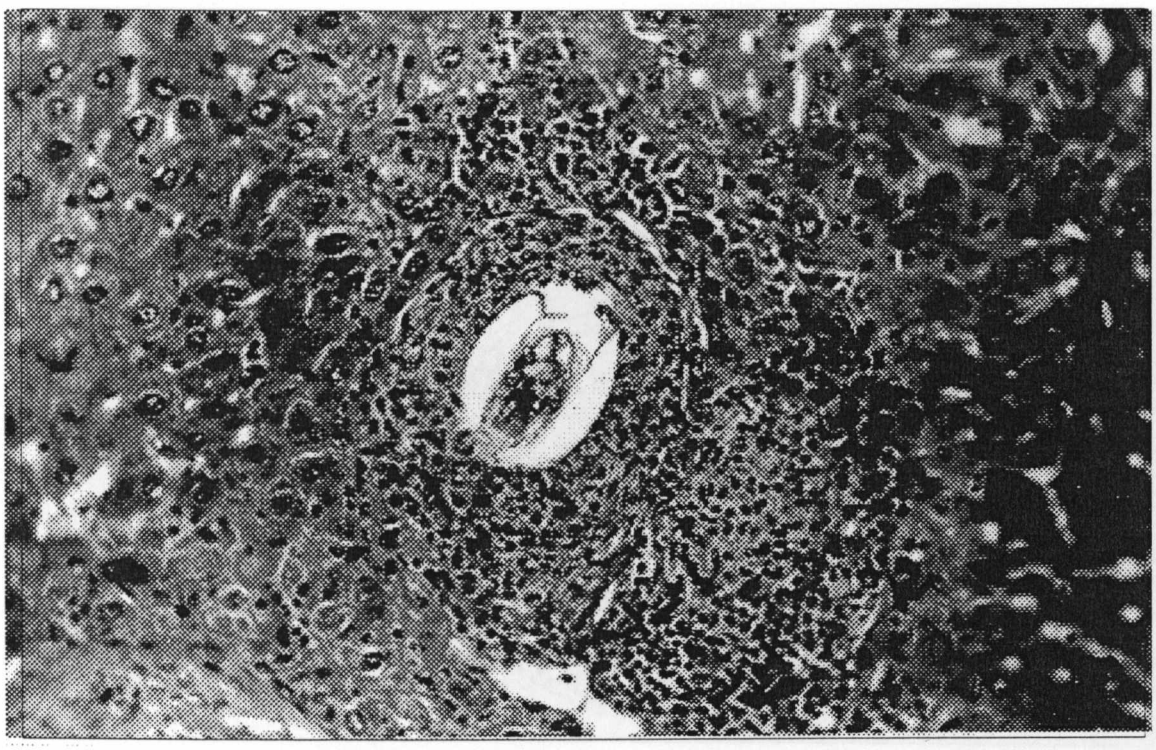

Fig 2: chronic phase periovular granuloma. Reaction around the egg is composed of macrophages and fibroblasts, with a few eosinophils. Central necrosis is absent. H & E, 200X.

for the presence of type I and type III collagens. The staining for fibronectin was less intense than that obtained for the acute phase. Orcein-positive elastic fibers, conspicously absent in acute and intermediate phase granulomas, appeared in a few granulomas of the chronic phase (Fig. 3). Desmin-positive cells increased their numbers proportionally from acute to chronic phase.

Granulomas formed around mature eggs in the intestine and lung showed a mixture of proliferative and exudative features, but they tended to maintain the same morphologic aspect when examined at different times of infection (Figs 4, 5). All components of extracellular matrix revealed by immunofluorescence microscopy in the liver were also present in granulomas of intestine and lung, although always in smaller amount (Fig. 7E, F).

Hydroxyproline content was evaluated only for the liver. Data expressed as μmol of hydroxyproline per gram of tissue versus number of eggs per gram of liver are depicted in Fig. 6A, B, C. Positive correlation occurred at the acute phase only. However, the concentration of hydroxyproline in the liver increased progressively with time of infection. The average numbers were: normal liver 3.42 μmol; acute phase 6.90 μmol, intermediate phase 7.84 μmol and chronic phase 8.77 μmol.

DISCUSSION

Present findings confirm and extend previous data which are suggestive that the so-called "immunological modulation" of schistosomal periovular granuloma is a morphological phenomenom peculiar to the liver (Grimaud et al. 1987, Vidal et al. 1992). Granulomas formed around eggs injected directly into the lungs (Lichtenberg 1962) or liver (Edungbola & Schiller 1979) differ accordingly with the previous immunological state of the host, that is, whether the recipient animal is already *S. mansoni* infected or not. However, injections of eggs into normal animals or even into those already infected (Domingo & Warren 1968), create an artificial and complex situation, with some peculiar features that differ from those of a cercarial infection, as has been discussed by Cheever et al. (1998).

Weinstock and Boros (1981, 1983) observed differences in size and cellular composition of granulomas formed at different intestinal segments. Probably these differences were small. Present data refer to the intestines as a whole, and no evidences of modulation were noted in that organ during different periods of infection.

Quantitative data about granuloma size and volume were subjected to morphometric evalua-

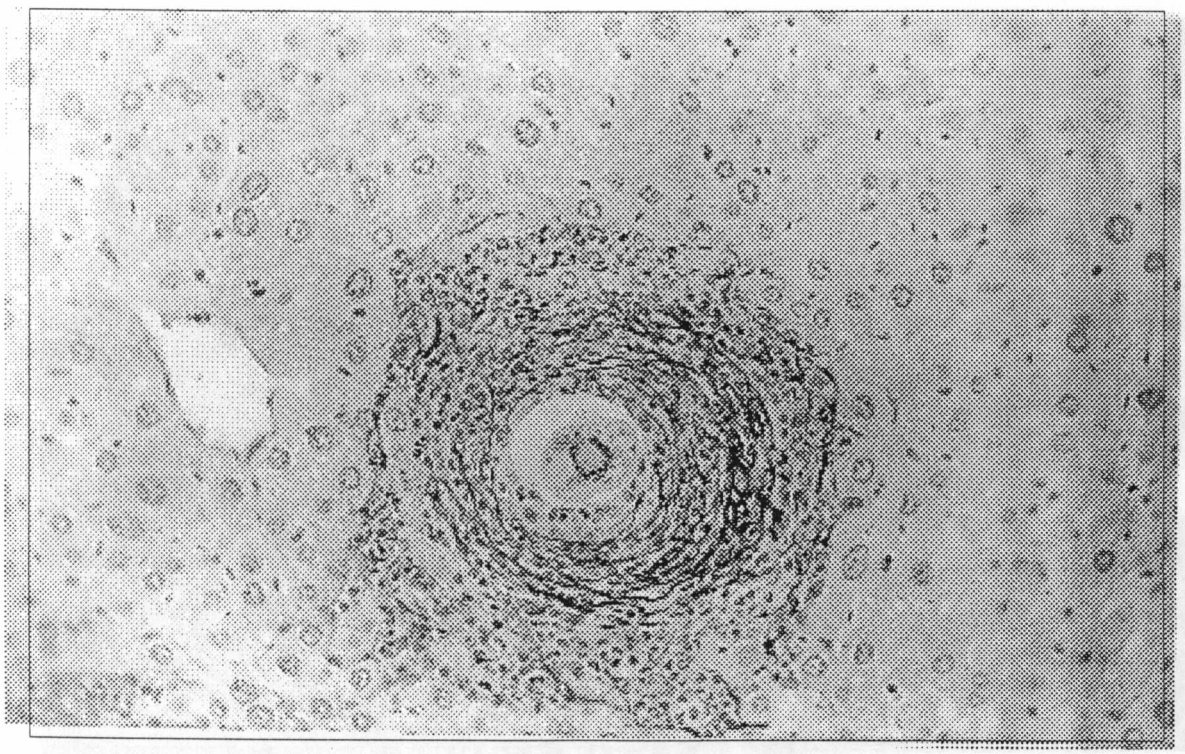

Fig. 3: a well delimited inflammatory reaction around a schistosome egg formed during the chronic phase of infection, exhibits fine, concentric, black, orcein-positive elastic fibers. Orcein method for elastic fibers, 200X.

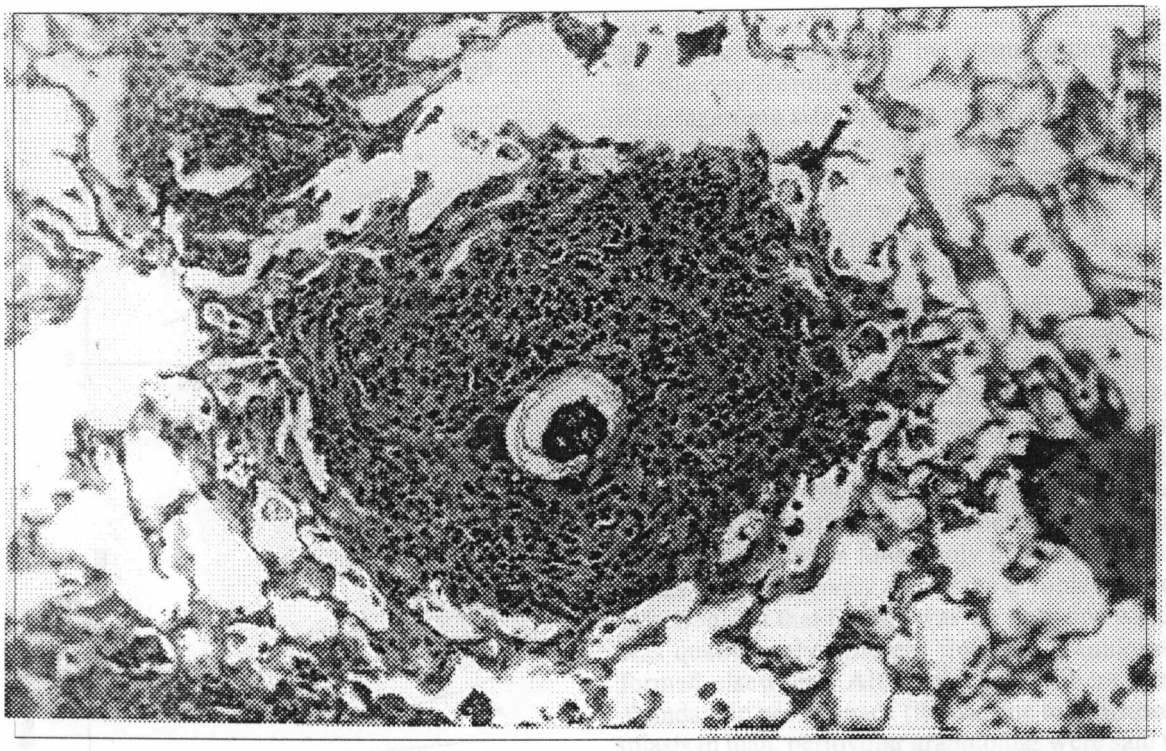

Fig 4: a mixture of exudative and proliferative features characterizes periovular granulomas formed within the pulmonary alveolar tissue. Intermediate phase of infection. H & E, 200X.

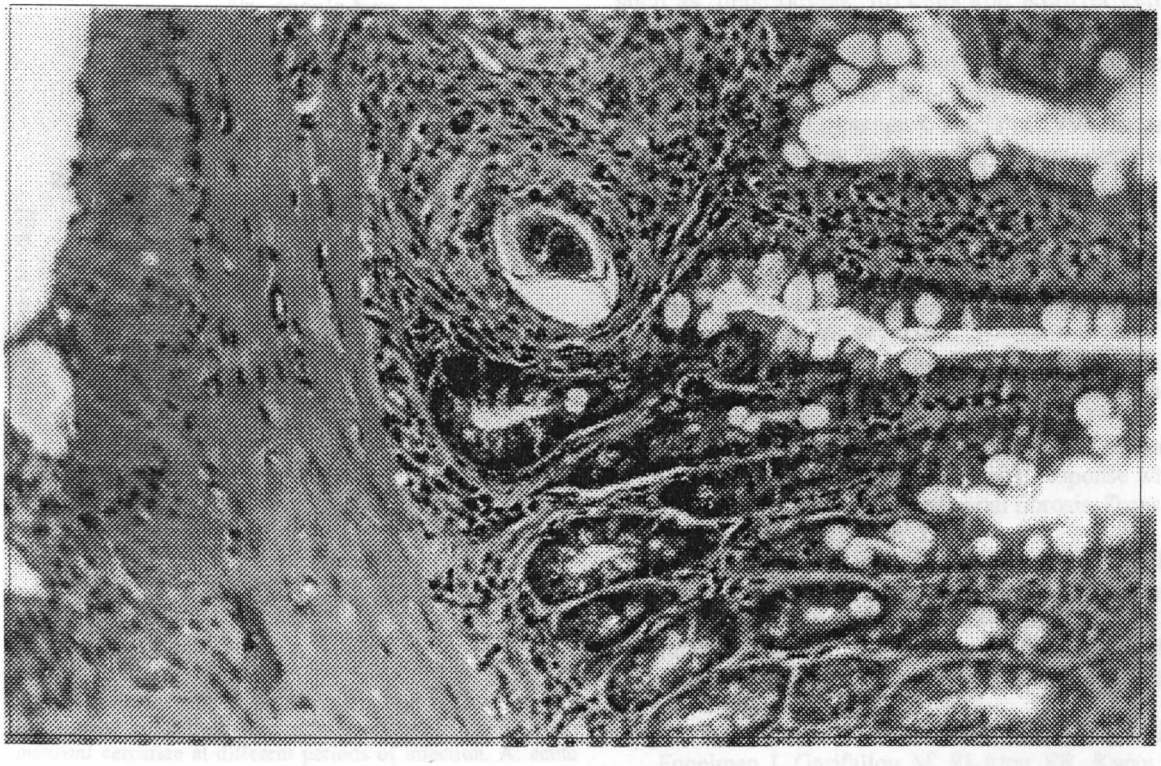

Fig 5: a small granuloma formed around a mature *Schistosoma mansoni* egg in the intestinal submucosa, during the acute period of infection. H & E, 200X.

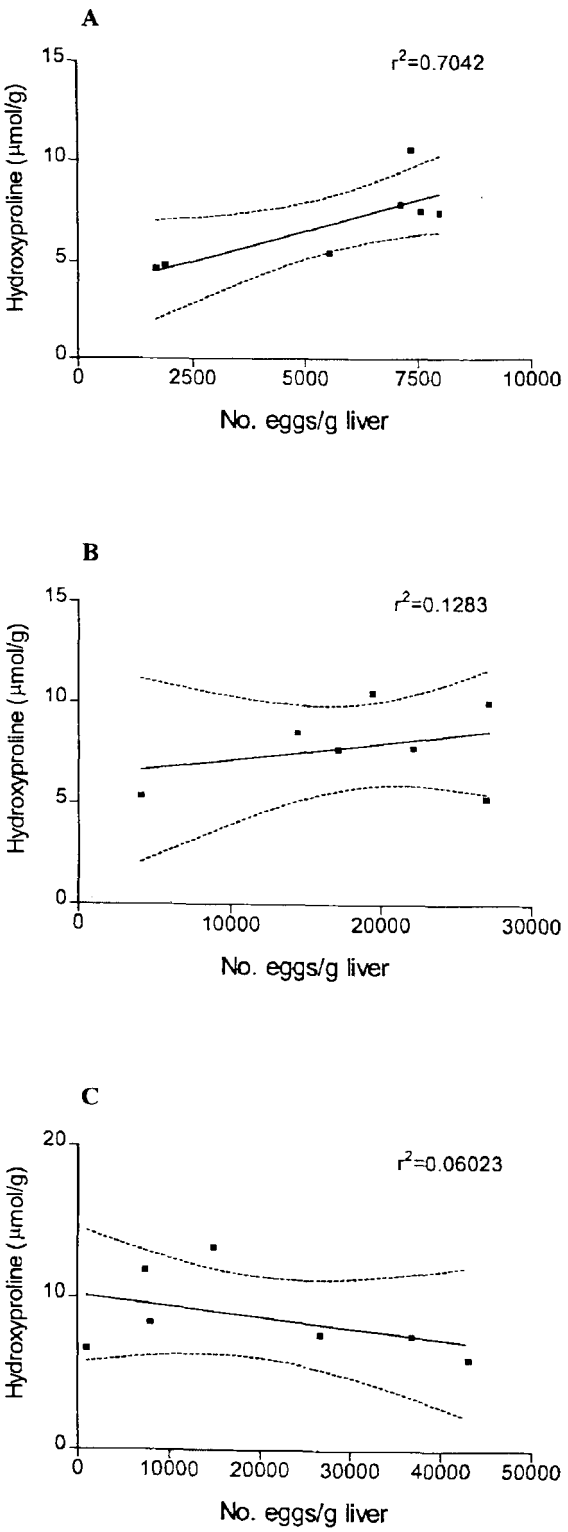

Fig 6: correlation between the number of eggs and hydroxyproline content in the liver of mice infected with 50 *Schistosoma mansoni* cercariae at different periods of infection. A: acute phase (8 weeks); B: intermediate phase (16 weeks); C: chronic phase (22 weeks).

tion, and the nature of granulomatous extracellular matrix was analyzed by immunofluorescent microscopy. Results indicated that changes, or lack of it, regarding size, appearance and cellular composition of granulomas located at different organs and at different times would mostly depend on local factors. The influence of general factors, such as immune cells, cytokine patterns and so on, is certainly important. However, more attention should be paid to the peculiar cellular population of the liver and the extracellular matrix it can produce and degrade. The matricial components which were investigated (Type I, III and IV collagens, fibronectin, laminin and proteoglycans) were also found in all granulomas and in all organs examined. However, their presence was much more prominent in the liver. As a matter of fact, elastic fibers (elastin) were found only in hepatic granulomas, and exclusively during the chronic stage of infection, but that finding was the only one which was qualitatively different from the granulomas formed elsewhere. Although elastic fibers were abundant in portal space fibrosis due to schistosomiasis in man, periovular granulomas were said to be devoid of such fibers (Junqueira et al. 1986, Andrade & Freitas 1991). Anyway, quantitatively, the large amount of extracellular matrix found in hepatic granulomas was the main change responsible for the morphologic aspects of modulation. Probably, the presence of special cellular elements, such as, hepatocytes, Ito cells and Kupffer cells, which are known to coordinately play an important role in formation and degradation of extracellular matrix in the liver (Blomhoff & Wake 1991, Casu et al. 1994, Gressner & Bachem 1994, Enzan et al. 1995) accounts for the peculiar way periovular granulomas are modulated in that organ.

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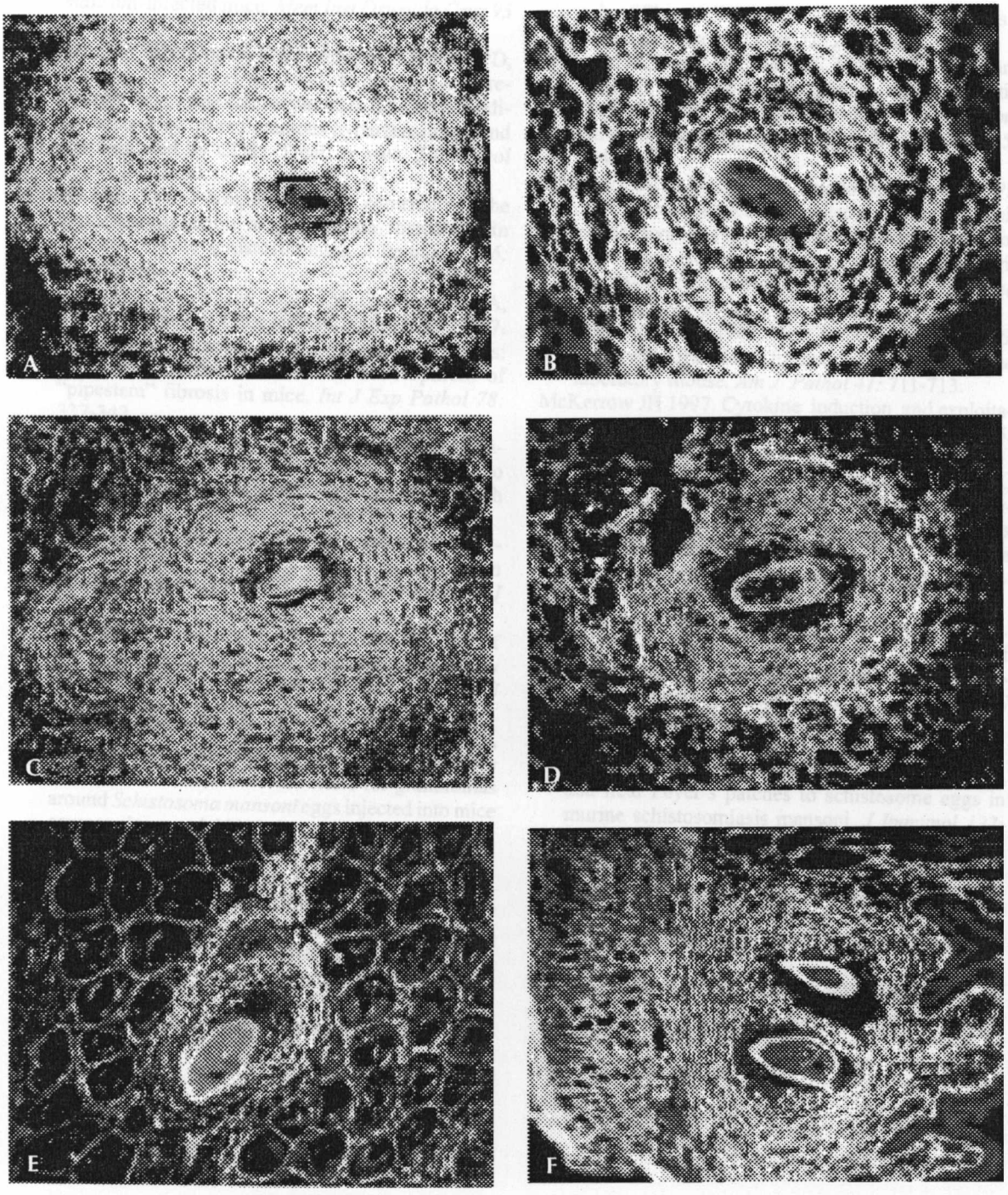

Fig 7: components of the extracellular matrix are depicted in the periovular granulomas by immunofluorescence. A: liver, type I collagen, acute phase of the infection, 200X; B: liver, type III collagen, intermediate phase, 400X; C: liver, fibronectine, acute phase, 200X; D: liver, laminine, acute phase, 200X. Note presence of blood vessel at the granuloma periphery (arrows) E: intestine, type I collagen, intermediate phase, 200X; F: intestine, fibronectine, chronic phase, 200X.

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Testing antifibrotic drugs in a new model
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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	108th

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

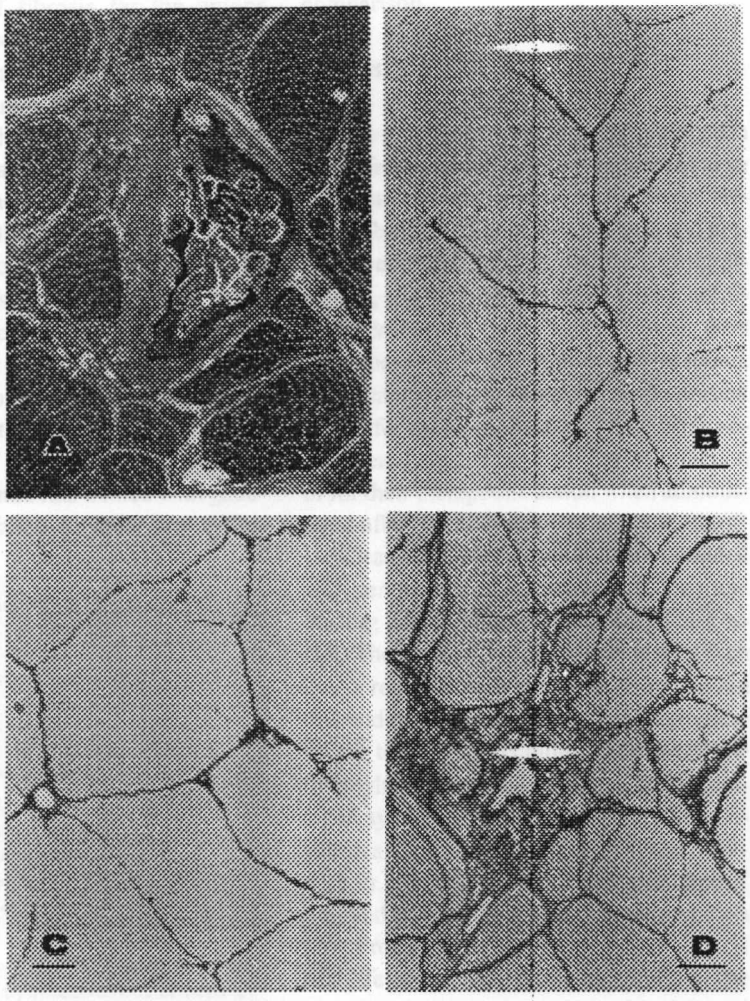

Figure 1 - A, Disintegrating adult worms (*Capillaria hepatica*) are seen at the center of the picture, surrounded by a necrotic-inflammatory reaction and a fibrous capsule. Septal fibrosis and regenerating hepatic nodules are also present. H & E, 120X (bar represents 100 µm). B, Representative of the 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	$12.50 \pm 3.87 \times 10^4$	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 ± 0.49*	Pentoxifylline (ip)
III	7.85 ± 1.54	Pentoxifylline (iv)
IV	6.41 ± 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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SHORT COMMUNICATION

Experimental Neuroschistosomiasis - Inadequacy of the Murine Model

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Neuroschistosomiasis is rarely observed in human pathology, but it is of considerable importance. To investigate its pathogenesis, consequences and response to treatment, an experimental model would be desirable, but is not yet available, in spite of a few indications of a suitable mouse model in the literature.

Severe, recent and late Schistosoma mansoni *infections in outbred and inbred strains of mice revealed widespread distribution of parasite eggs in several organs, but only exceptionally did eggs reach the encephalus, thus revealing the inadequacy of the mouse as an experimental model for neuroschistosomiasis.*

Key words: neuroschistosomiasis - *Schistosoma mansoni* - murine model

Neuroschistosomiasis can be a serious complication for patients infected with *Schistosoma mansoni*. The parasite eggs can reach the central nervous system either by embolization or by abnormal migration of adult worm pairs. Worms may pass through Batson's plexus, migrating from the portal to paravertebral veins, helped by retrograde blood flow generated by an eventual and sudden increase in abdominal pressure. It has been suggested that deposition of immune complexes, especially at the choroid plexus, can also result in central nervous system involvement in schistosomiasis (Pitella & Bambirra 1989). However, severity of clinical manifestations is certainly related to the presence of adult worms and their eggs, which correlate with the signs and symptoms of myelitis, radiculitis, tumor and meningeal irritation attributed to neuroschistosomiasis (Andrade 1986). In a few human cases, worms and eggs have been found in leptomeningeal veins in the brain and spinal cord, especially at the lumbosacral and thoracic regions, associated with phlebitis, arteritis, and chronic granulomatous inflammation (Gama & Sá 1945, Pondé et al. 1960, Ferreira et al. 1998). Due to difficulty in obtaining pathological material, diagnosis of neuroschistosomiasis in the large majority of cases relies on positive serology in the liquor and/or suggestive imaging (Ferrari et al. 1995, Ferrari 1999). Considering the important problems posed by neuroschistosomiasis, it is surprising that only a few attempts have been made to experimentally study it. The present investigation was conducted to find out whether the murine model would be suitable for such study. The literature has very little information concerning neuroschistosomiasis in mice. Aloe et al. (1996) described finding schistosome eggs in histological sections from the brain of mice and reported that mice with *S. mansoni* periovular granulomas in the brain showed decrease of nervous growth factor expression. Based on such findings, Fiore et al. (1996) reported that mice infected with *S. mansoni* exhibited behavioral disturbances, probably associated with modifications in the levels of nerve growth factor and cytokines induced by granulomas. These two reports did not indicate how frequently *S. mansoni* eggs reach the central nervous system of the mouse. In the present report, we attempted to do that, as follows: (1) outbred Swiss mice, 20-25 g, of both sexes, were infected with either 30 or 50 *S. mansoni* cercariae, Feira de Santana strain (Andrade & Sadigursky 1985) by the transcutaneous route. For early infection, animals were sacrificed at 8-10 weeks of cercarial exposure. For late infection, animals with 30-31 week old infection were sacrificed; (2) inbred BALB/C mice, 15-18 g, of both sexes, were infected with 30 cercariae. When the infection was proved patent by the presence of viable eggs in the stools, these animals were submitted to reinfection five times with 15 cercariae once a week. This procedure increases the severity of schistosomiasis, with higher probability to develop pipe-stem fibrosis in the liver (Araújo Santos et al. 2000). Mice were sacrificed at 31, 40 and 52 weeks after first cercarial exposure; (3) inbred C57 mice were infected with 20 cercariae, re-infected with 10 cercariae, and sacrificed at 40 weeks following the first cercarial exposure.

For all animals sacrificed, except C-57 and 5 BALB/C mice, the liver, lung, small and large intestines, pancreas, kidney and spleen were removed and weighed. Fragments of these organs were digested in 60 ml of a 4% potassium hydroxyde solution for the counting of eggs, according to Cheever (1970) (Table I). For the encephalus, the entire organ was always digested and the samples for the microscopic seach of eggs were collected after sedimentation for several hours. Only a few schistosome eggs were found in the nervous tissue (Table II). The chance of find-

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TABLE I

Average number of *Schistosoma mansoni* eggs per gram of tissue obtained from several organs of mice

Organs	Simple Infection (50 cercariae) 8-10 week 10 animals Outbred Swiss	Simple Infection (30 cercariae) 30 week 10 animals Outbred Swiss	Re-infection 31 week 7 animals BALB/C	Re-infection 52 weeks 12 animals BALB/C
Lung	1,418 ± 344	2,415 ± 4,344	1,309 ± 3,465	58 ± 110
Liver	11,251 ± 942	2,085 ± 1,559	7,030 ± 3,991	5,420 ± 9,461
Spleen	605 ± 109	-	358 ± 820	0 ± 0
Small intestine	22,357 ± 27,376	4,301 ± 7,239	4,638 ± 4,717	10,062 ± 20,377
Large intestine	52,38.5 ± 9,047	1,361 ± 3,765	-	120 ± 197
Pancreas	5,993 ± 12,494	257 ± 362	-	2,048 ± 4,385

TABLE II

Presence of *Schistosoma mansoni* eggs in the encephalus of mice as seen after potassium hydroxide digestion of the whole organ

Groups	Infection	Duration (weeks)	No. of animals	Positives	%	No. eggs
Swiss	Single (50c)	8-10	10	0	0	0
Swiss	Single (30c)	30	10	1	10	1
BALB C	Re-infection	31	7	0	0	0
BALB C	Re-infection	40	5	2	40	2 and 7
BALB C	Re-infection	52	12	2	17	1 and 3
C57	Re-infection	40	12	2	17	1 and 4

ing eggs somewhat increased after prolonged and more severe infections. These results are difficult to reconcile with those of Aloe et al. (1996). They found between 20 and 50 schistosome eggs per mouse after examining 40 frozen brain sections. We did not succeed in finding eggs in histological sections of the brain, and because of that we resorted to the digestion method of the whole encephalus. Fragments of the liver were fixed in formalin and 5 µ-thick paraffin sections obtained were stained with hematoxylin and eosin, and sirius-red for collagen. The majority of the animals showed pipestem fibrosis, especially those of re-infection groups.

In conclusion, our results indicate that mice with severe infection, with widespread distribution of schistosome eggs in several organs, failed to show significant involvement of the central nervous system. Therefore, the murine model that has been so successfully applied to investigate multiple aspects of human schistosomiasis did not appear to be suitable for experimental studies about neuroschistosomiasis.

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Chemotherapeutic effects on larval stages of *Schistosoma mansoni* during infection and re-infection of mice.

Efeitos da quimioterapia nos estágios iarvais do *Schistosoma mansoni* durante infecção e re-infecção de camundongos

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Abstract *The sensitivity of the larval stages of* Schistosoma mansoni *to chemotherapy with praziquantel and oxamniquine was tested in mice during primary and secondary infections and after different intervals from cercarial exposure. Worm recovery by perfusion of the porto-mesenteric system, followed by counting and a morphometric study of the parasite, allowed the conclusion that the relative resistance of the larval stages of* S. mansoni *to schistosomicide drugs, demonstrated in primary infections, also persists when the host is already infected..This indicates that a therapeutic "failure" may result when an infected host is treated some time after being re-infected, because of the presence of migrating, drug-resistant, immature forms of the parasite.*
Key-words: Schistosoma mansoni. *Larval stages. Praziquantel, Oxamniquine.*

Resumo A susceptibilidade dos estágios larvais do *Schistosoma mansoni* aos esquistossomicidas praziquantel e oxamniquine foi testada em camundongos durante infecção primária ou secundária, e após diferentes intervalos de tempo após a exposição cercariana. A avaliação foi feita pela contagem dos vermes após recuperação destes por perfusão do sistema porto-mesentérico e pelo estudo morfométrico dos mesmos. O estudo revelou que a relativa resistência das formas larvais aos esquistossomicidas, já demonstrada em infecção primária, persiste no caso de hospedeiros já infectados. Este fato indica que uma "falha" terapêutica pode resultar quando o tratamento é feito em hospedeiros re-infectados recentemente, em virtude dos mesmos apresentarem formas migrantes e imaturas do parasita, as quais são particularmente resistentes aos esquistossomicidas.
Palavras-chaves: Schistosoma mansoni. *Estágio Larval. Praziquantel. Oxamniquine.*

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Oxamniquine and praziquantel are the two drugs used in Brazil for the treatment of schistosomiasis. Large scale treatment programs using either drug have been carried out in several Brazilian endemic areas[10 11 13]. Treatment efficacy is considered satisfactory, but "failures" have been registered with variable frequency. Such therapeutic failures are often attributed to the development of drug resistance by the worms and to early post treatment re-infection[2 6 7]. However, in areas of high transmission, another possibility may occur. Treated individuals, even cured, may re-establish the infection in consequence of the existence of migrating larval forms of *Schistosoma mansoni*, which are usually resistant to chemotherapy, as experimentally demonstrated[16 21].

Sabath et al[16] have shown that schistosomula are far less susceptible than adult worms to six schistosomicides at curative doses, including praziquantel and oxamniquine. However, these studies were performed in mice with experimental primary infections treated at different time points after cercarial exposure. Since the effect of chemotherapy is influenced by the immune state of the host[3 4 15], the response of the larval stages of *S. mansoni* to schistosomicides should also be tested during re-infection.

The present investigation studied the effects of oxamniquine or praziquantel on different larval stages of *S. mansoni* in mice with primary infection compared to mice submitted to re-infection.

MATERIAL AND METHODS

Two main experiments were performed: The first experiment involved 185 outbred Swiss mice of both sexes, weighing 18-22 g, that were infected transcutaneously with 100 *S. mansoni* cercariae of the Feira de Santana strain[1]. This strain has been maintained through successive passages in laboratory-raised *Bimphalaria glabrata*. The infected animals were randomly separated into 3 groups:

Group 1: consisted of 25 mice that were not subject to drug treatment, and served as untreated controls. Animals were sacrificed 40 days after cercarial exposure, when the worms were recovered from the portal-mesenteric venous system by perfusion, according to Duvall and DeWitt [8].

Group 2: included 80 mice treated with oxamniquine. The drug was suspended in distilled water and administered by gavage in a single dose of 100mg/kg/bw. at 5, 10, 20 or 30 days following infection, 20 different animals being treated at each time point. Thirty days after treatment (35-60 days after infection), the animals were anesthetized and sacrificed by severing of the abdominal aorta. Worms were recovered by perfusion of the portal system.
Group 3: also with 80 mice, received praziquantel, administred by gavage in a dose of 400 mg/kg/w, otherwise following the same steps as for the previous Group. Another approach was made with 153 Swiss mice, which were at first submitted to transcutaneous infection with 30 *S. mansoni* cercariae. Thirty to forty-five days afterwards, the animals were exposed to re-infection with 50 cercariae. Only mice passing viable eggs in the stools were used. Similarly to the first experiment, re-infected animals were separated into 3 groups, as follows: a) untreated re-infected controls (*n* =13); b) treated with oxamniquine (100 mg/kg, n=64); and c) treated with praziquantel (400 mg/kg, n =64). Chemotherapies were administered by gavage one time only, 10, 20 or 30 days following re-infection, for sub-groups of animals, according to the intervals after re-infection. Twenty days after treatment, re-infected animals were anesthetized and sacrificed for recovery of worms. Untreated controls were sacrificed 30 days after re-infection.
An additional Group was also included in this experiment. It was one extra non-treated control group with primary infection for comparing the effect of the cumulative infections (*n*=12, 30 cercariae).

Drug efficacy was determined through the percentage of worm load reduction, by using the formula by Sabah et al[15] (R*=100 – (t x100/c), where t = median of worm recovered from the treatment group and c = median of worm recovered from the untreated group, and comparing treated and non-treated animals in their respective groups and sub-groups. Median numbers of worms were compared by means of the Mann Whitney test.

In an attempt to separate the "young" and "old" worms from infections made at different times, a morphometric analysis was performed. Male and female worms were recovered from mice with one single infection, and from those infected and re-infected, including treated and non-treated controls. The worms were fixed in neutral 10% formalin, stained with carmin chloride, dehydrated in alcohol, cleared in creosote and mounted on glass slides with a 1:1 mixture of Canadian balsam and creosote for microscopic examination. Considering sexual dimorphism, morphometric evaluation was made separately for male and female worms. A Leica Quantimet 500C (Leica Cambridge, U.K.) system, with a Sigma-Scan Measurement semi automatic morphometric device (Jandel Scientific, SF, USA) was used. Corporeal length, number of testicular masses and the presence of eggs in uterus were the parameters considered.

RESULTS

The first experiment revealed that the sensitivity of the *S. mansoni* larvae to both oxamniquine and praziquantel varied according to their developmental stage. Oxamniquine yielded poor results during the first 20 days of infection, the percentage reduction of worm-load was 28-34% in treated as compared to non-treated mice (Table 1). However, 30 days after exposure, the percentage reduction was 86 to 100%, **essa redução foi estatisticamente significante quando comparada aos períodos anteriores do tratamento. Nos demais perídos o efeito do quimioterapico foi semelhante.** As for praziquantel the efficacy was 48 to 52% for the animals treated until 10 days after cercarial exposure, dropping to 19% on the 30th day. **Nos primeiros dois pontos do tratamento o praziquantel não demonstrou melhora na capacidade de redução carga parasitária (p= 0.9440). Entretanto houve variações significativas a partir do 10 dias até o 30 dia pós infecção.** Compared to oxamniquine, the efficacy of praziquantel **did not essentially differ (entre o 5 e o 10 dia pós infecção),** although the latter seemed more effective at the 20th day of infection, while better effects occurred in the former by the day 30 (Table 1).

During the second experimet compared to the re-infected controls, the percentage reduction of worm load was greater for both treatment groups. (Figure 1 and Table 2). Oxamniquine was seen to be very effective by the 10th day following re-infection, presenting a reduction of 64%. These figures decreased subsequently **no 20 dia pós infecção (p=0.0409)**, still varying with time, although a few mice were seen totally cured among those treated later on**, não demonstrando variação do efeito quimioterápica entre 20 e 30 dias pós exposição cercariana (p= 0.1031).** As a matter of fact, reduction of worm-load for the animals re-infected and treated reached 75%. **Embora** During re-infection praziquantel was more effective than oxamniquine in reducing worm load. A 76% reduction occurred when treatment was given in the 10th, 20th (74%) and 30th day following re-infection (76%). **O efeito do praziquantel foi semelhante nos três momentos estudados. Não houve diferenças significantes na ação dos quimioterápicos nos três pontos estudados.** All animals treated after re-infection showed statistically significant

differences in worm-load reduction when compared with the re-infected but untreated control group.

The average male worm length for the control group of single infection was 7,958 x $10^{-3}\mu m$, with a confidence interval between 7,644.5 and 8,272.2 $x10^{-3}$. The figures for females were: 10,457.4 x 10^{-3} with confidence interval of 9,536.1 to 11,378.7 x 10^{-3}. To evaluate worm length from a second infection, the values obtained were distributed in quartils. In quartile 25 it was noted that the length of male and female worms was 6,300 x 10^{-3} and 7,356 x 10^{-3}, respectively. It was considered that worms, male or female, with body length equal or inferior to these figures represented worms from the second infection. Figure 2 is based on such data.

DISCUSSION

The present study shows that the effect of the schistosomicide drugs, praziquantel and oxamniquine, upon migrating schistosomula in mice differs according to the drug used and the time of infection. As a matter of fact, several authors remarked that the larval stages of *S. mansoni* are less susceptible to chemotherapy than young and adult worms[3 16 21]. These differences were now seen to be accentuated when the treatment was made after several different periods following re-infection.

It has been demonstrated that the drug-induced killing of adult worms is mediated by the host immune system[3 4 15]. The schistosomicides administered cause focal areas of damage to the worm tegment[12 18], exposing target antigens to host antibodies, which ultimately provoke the worm death[3 9 17]. Unfortunately, the role played by the immune system cannot be enhanced by means of artificial antigenic stimulation[19].

There are few and contradictory experimental data concerning the participation of

Decreased humoral and pathologic responses in undernourished mice infected with *Schistosoma mansoni*

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Abstract

It has been demonstrated that a low-protein diet interferes with the morphology of hepatic lesions resulting from *Schistosoma mansoni* infection in mice. Periovular granulomas are smaller than in controls, and portal pipestem fibrosis does not develop. Here we compared the humoral and cellular immune responses in well-nourished and undernourished mice during chronic *S. mansoni* infection. Splenocyte proliferative response against *S. mansoni* soluble egg antigen (SEA) or concanavalin A (Con A) was similar for both groups. The production of IFN-γ, IL-4 and IL-5 could only be detected in splenocyte cultures stimulated by Con A. But no difference was observed between the two groups of mice. Undernourished mice produced detectable levels of anti-SEA antibodies when compared to non-infected undernourished animals. However, the serum levels of SEA-specific IgG1, IgG2b and IgG3 antibodies in infected mice were significantly higher in well-nourished in comparison with undernourished mice. Undernourished animals also exhibited diminished periovular granuloma size and less portal fibrosis than well-nourished infected controls. These results support the importance of the host nutritional status on the humoral immune response in mice and its effects on the development of periovular granulomas in malnourished animals infected with *S. mansoni*.

Introduction

Schistosomiasis mansoni is an important world health problem, especially in developing countries, where it is usually associated with an impaired nutritional status. The severe form of the disease (hepatosplenic form) results from the immuno-inflammatory response against schistosome eggs deposited in intrahepatic periportal veins, causing the development of periportal fibrosis and liver malfunction (Warren, 1966). Although *S. mansoni* infection aggravates the nutritional status in human beings, the pathological manifestations of schistosomiasis in undernourished individuals appear less severe than in to well-nourished patients (Coutinho, 1972, 1997).

An experimental model to induce mal nutrition in mice infected with *S. mansoni* has been developed in our laboratory, feeding Swiss Webster mice with a hypoproteic diet, referred to as "regional basic diet" (RBD) wich is related to food habits of poor income people residing in endemic area of Northeastern Brazil (Coutinho, 1980; Coutinho et al, 1997). In this situation undernourished mice develop smaller peri-ovular granulomas but not the periportal fibrosis observed in about 40% of well-nourished infected controls (Andrade & Cheever, 1993; Coutinho et al, 1997).

Fibrosis development is a result of a balance between collagen deposition and its degradation by catalytic enzymes (Burt, 1993). These phenomena are regulated by immune responses through the production of soluble mediators which affect different cell types stimulating or inhibiting both the production of collagen and of collagenases. In *S. mansoni* infection, modulation of granuloma formation and collagen deposition are associated with a Th2 response stimulated by the production of schistosome egg antigens (Andrade & Warren 1964; Wynn & Cheever, 1995). Treatment of *S. mansoni*-infected mice with interferon (IFN-γ) causes a decrease in granuloma size and fibrosis deposition (Csaja et al,

1989). Since immune responses are usually depressed in undernourished individuals (Machado, 1983; Chandra, 1997), this paper deals with the importance of the host's nutritional status on the immune response to schistosome antigens in undernourished mice chronically infected with *S. mansoni* and its influence on the development of periovular granulomas and hepatic fibrosis in these animals.

Materials and Methods

Animals

Three week-old Swiss Webster mice (male and female) were raised and maintained at the animal facilities of the Aggeu Magalhães Research Center Oswaldo Cruz Foundation (Recife, Brazil). Mice were submitted to a deficient diet (RBD) planned to simulate the one usually ingested by low income people in Northeast Brazil (Coutinho et al., 1997), with 8% protein content. Control mice (well nourished groups) were fed a balanced, commercial mouse chow (Nuvital Nutrients Ltda, Colombo, Parana, Brazil), containing 22% protein. Diets were given in pellet form.

Experimental infection

Mice were submitted to percutaneous infection with 30 recently shed *S. mansoni* cercariae obtained from laboratory-raised *Biomphalaria glabrata* (Belo Horizonte strain). The animals were distributed into following groups:

group 1 – undernourished

group 2- infected, undernourished

group 3- well-nourished

group 4- infected, well nourished.

Animals were fed their respective diet 30 days before infection up to the end of the experiment. Infection was confirmed in each mouse by detection of *S. mansoni* eggs in the feces 50 days after cercarial exposure.

Parasitologic and histopathological studies

Sixteen weeks after infection, the animals were sacrificed. Liver and intestine were removed and weighed. Pieces of both organs were placed into 4% potassium hydroxide for

egg counting (Cheever, 1970). Samples of liver tissue were fixed in Bouin's fixative and/ or Millonig formol for histopathological studies after paraffin embedding, 5μm-thick sections were stained with haematoxylin/eosin or with picrosirius-red method for collagen (Junqueira et al. 1979).

Morphometric studies

Randomly sampled 5 μm-thick liver sections stained with picrosirius-red were examined by semiautomatic morphometry using the Q500MC Image Processing and Analysis System (Leica Cambridge, Cambridge, England). A total sectional area $1.34x10^6 \mu m^2$ per animal was evaluated by examining all periovular granulomas present in the section. The following granuloma parameters were calculated: size, volume, volume density and numerical density. The granuloma volume density was calculated as the quotient of the total granuloma profile area to the total sectional area studied per animal. The number of granulomas per unit volume of liver was assessed by applying the Weibel's formula (Weibel, 1969). The sectional area of the fibrous tissue, red stained, was directly measured and calculated as a percentage of the total area examined.

Collagen assessment

The method of Bergman and Loxley (1963) was used to determine collagen concentration by the measurement of hydroxyproline content. Briefly, after fixation in neutral 10% formalin a liver fragment of 100-250 mg were hydrolyzed for 18h in hydrochloric acid (6 N) at 110°C, neutralized in NaOH (10N) and HCl (3N). Samples of 100 μl were treated in mixture of chloramine T and Erlichs' reagent and read in a Hitachi 200 spectrophotometer (558nm).

Spleen cells culture

Single cells suspensions were prepared from spleens aseptically removed from mice of the four experimental groups. Spleen cells suspensions were prepared in RPMI medium (Life Technologies, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% FCS (Hyclone, Logan, Utah), L-glutamine (2 mM), vitamins, sodium pyruvate (1 mM), Hepes (10 mM), $5x10^{-5}$ M of 2-mercaptoethanol, and gentamycin (50 μg/ml) (Sigma). For cytokine determinations, spleen cells were cultured in 24 well plates and stimulated with 1 μg/ml of concanavalin A (Con A) (Sigma) or *S. mansoni* egg antigen (SEA) (10 μg/ml). Cell-free supernatants were collected after 72 hours of incubation and stored at -20°C for cytokine analysis. To evaluate the proliferative response, splenocytes were plated in 96-well plates at $4x10^5$/well in 200 μl and triplicate wells were stimulated with Con A or SEA for 72 hours, as described in figure legends. After pulsing with 1 μCi of [*methyl*-^{3}H] thymidine (Amersham, Little Chalfont, England) for 12 hours, proliferation was assessed by measurement of ^{3}H thymidine uptake in a β-plate counter (Packard, Meriden, Connecticut).

Cytokine and antibody determinations

Levels of IFN-γ, IL-5 and IL-4 in culture supernatants were determined by Sandwich ELISA, using antibody pairs and recombinant cytokines from PharMingen, following manufacturer's instructions. Reaction was developed using the 3,3', 5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Kinkergaard & Perry Laboratories, Gaithersburg, MD) and read at 450nm.

Anti-SEA isotype production was evaluated by ELISA using microtiter plates (Nunc-MaxiSorp plates) coated overnight at 4 °C with 3 μg/ml SEA in 0,02 M sodium carbonate pH 9.6. After incubation for two hours at room temperature with sera from different experimental groups, biotinylated isotype-specific anti-mouse IgG1, IgG2a, IgG2b or IgG3 antibodies (PharMingem) were added for 45 min, followed by streptoavidin-peroxidase conjugate for 30 min (Sigma). Reaction was developed using TMB substrate, as described above.

Statistical analysis

Data were analyzed using Student's *t* test or Kruskal-Wallis non-parametric test, as indicated in the text. Differences were considered significant when $P < 0.05$. The analyses had been carried through with the aid of Prism 3.0 and Microsoft®Excel 1997 .

Results

Egg granulomas and liver fibrosis development

Four months after *S. mansoni* infection, the livers of undernourished mice had a reduced number of granulomas per liver area when compared to well-nourished mice (Table 1). In undernourished mice, egg-granulomas were smaller than those observed in liver sections of well-nourished animals (Table 1 and Figure 1). *S. mansoni* infection caused an increase in collagen content in the livers of undernourished and well-nourished mice (Figure 2). However, undernourished infected mice had significantly less liver collagen than well-nourished ones (Figure 2; $P<0.001$).

Lymphoproliferative response in *S. mansoni*-infected mice

In order to investigate a possible contribution of the immune responses of infected mice to the development of fibrosis in *Schistosomiasis mansoni* the lymphoproliferative responses of mice from different groups were compared, four months after *S. mansoni* infection (Figure 3). Spleen cells from infected mice of both groups had similar proliferative responses to Con A when compared to uninfected mice (Figure 1A). The proliferation index of spleen cells of undernourished infected mice stimulated in vitro with Con A was also similar to that of well-nourished infected mice (Figure 1A). In the four experimental groups, the proliferative responses of splenocytes to SEA were low and differences observed were not statistically significant (Figure 1B).

Cytokine production by spleen cells after *S. mansoni* infection

The production of Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines was evaluated four months after *S. mansoni* infection. Spleen cells from both undernourished and well-nourished infected mice produced low levels of IFN-γ upon stimulation with Con A and undetectable levels of this cytokine upon stimulation with SEA (Table 2). The IL-5 levels produced by spleen cells from undernourished and well-nourished infected mice after Con A and SEA stimulation were also similar (Table 2). IL-4 production was not detected in cell cultures from mice in any experimental group.

SEA-specific antibodies

Four months after *S. mansoni* infection the isotype profile of undernourished and well-nourished infected mice. The levels of SEA-specific IgG1, IgG2a, IgG2b and IgG3 antibodies detected in the serum of undernourished infected mice were 2 to 4-fold lower as compared to those of well-nourished infected controls (Figure 5; $P<0.05$).

Discussion

According to previous investigators, both cellular and humoral immune responses may be affected by the host's nutritional state (Beisel, 1987; Cunnighan-Rundles, 1982; Chandra, 1999). Results presented here show that undernourished infected mice have similar proliferative and cytokine responses when compared to well-nourished infected animals, but produce antibody levels significantly lower than those of well-nourished infected controls. This type of immune response is associated with a less severe pathology in undernourished mice (granulomas reduced in number and size) when compared to controls.

In a study carried out by Ghanem et al. (1987), patients with the hepatoesplenic clinical form of schistosomiasis had an increased production in the IgG synthesis and higher levels of circulating immune complexes. The demonstration of IgG deposits in the Disse space in liver sections of patients with advanced Symmers' fibrosis caused by *S. mansoni* infection (Grimaud et al, 1977) suggests that overproduction of SEA-specific IgG antibodies contributes to the aggravation of the pathological process. Thus, it is possible that the higher levels of antibodies found in infected well-nourished mice contribute to a higher collagen deposition in liver sections observed in these mice, in comparison with undernourished animals.

Some published papers have demonstrated that the host's nutritional status also affects the biology of the parasite (Oliveira et al., submitted for publication; Neves et al., 2001). Worms recovered from undernourished infected mice show morphological changes in their internal and external structures and exhibit a low fecundity when compared to parasites recovered from well-nourished hosts (Oliveira et al., submitted for publication;

Neves et al., 2001). In low-protein fed mice, the low fecundity detected in these parasites probably affects the production of eggs and of periovular granulomas, as well as the progression to the liver periportal fibrosis (Coutinho, et al., 1997).

The proliferative response of spleen cells obtained from well-nourished and undernourished mice to SEA was low. Similarly, the levels of IFN-γ were low or undetectable upon stimulation with Con A and SEA, respectively. These findings are in agreement with data in the literature showing suppression of IFN-γ, an antifibrosant cytokine, and proliferative responses in chronic *S. mansoni* infection (Cheever et al, 2000; Fallon 2001). It is likely that the modulation of the immune response may induce alterations in the size, shape and composition of periovular granulomas (Andrade & Warren, 1964). Concomitantly, the fibrotic reaction in the liver is accelerated by an increase in collagen deposition (Grimaud et al, 1977) and linked to a Th2 response (Yamashita & Boros, 1992; Cheever et al, 1994; Chiramonte et al, 1999).

It is well known that malnutrition has several effects on mechanisms of repair, such as decreased fibroblast proliferation, collagen and albumin production and remodeling of wound (Mora, 1999). Recent data from our laboratories suggest that the host's nutritional status plays a role in connective tissue changes of hepatic schistosomiasis in mice (Coutinho et al., submitted for publication). Therefore, the decreased fibrosis observed herein in undernourished infected mice may result from an impaired mechanism of repair, a lower antigen load or a low antibody production due to a low protein synthesis in a low-protein fed host.

The finding that undernourished mice develop less severe disease upon *S. mansoni* infection may seem paradoxical if one takes into account that undernourished infected hosts

usually present well prominent injuries upon infection with other aggressive agents. However, in those cases, the injury results from the higher capacity of proliferation of the infectious agent, a fact that does not occur in schistosomiasis mansoni, where the parasites do not multiply in the host. In the case of *S. mansoni* infection, the tissue pathology result from an exacerbated immune response, which may be depressed in situations of low protein intake.

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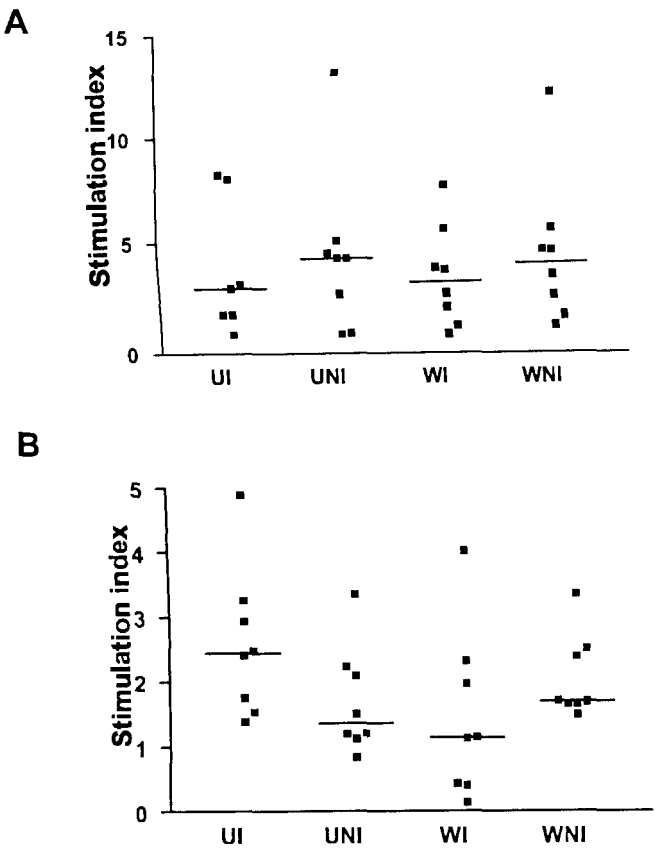

Figure 1: Comparison of lymphoproliferative response of spleen cells from *S. mansoni*-infected mice. Spleen cells from undernourished infected (UI) or noninfected (UNI) mice and well-nourished infected (WI) or noninfected (WNI) mice were obtained 4 months after *S. mansoni* infection. Splenocytes were stimulated in vitro with Con A (A) or SEA (B). Lymphoproliferation was assessed by measurement [^{3}H] TdR uptake after 72 hours of cell culture. Stimulation index of spleen cells cultures from individual mice (8 per group) were calculated by the ratio between stimulated and non-stimulated wells. (P> 0.05)

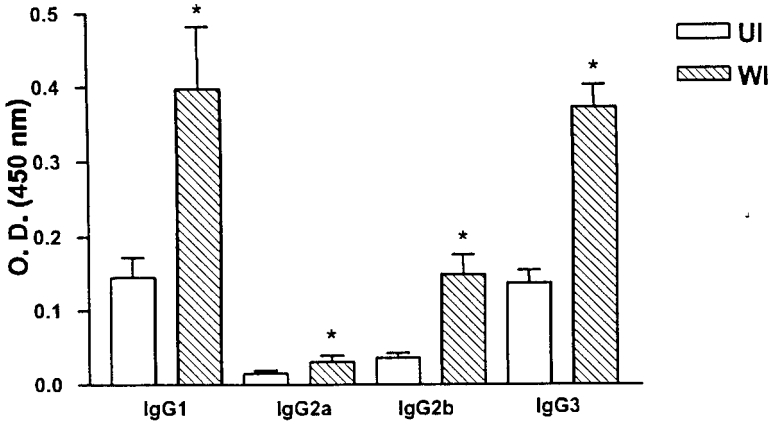

Figure 2: Titers of SEA-specific antibodies in *S. mansoni*-infected mice. Sera from infected mice fed a low-protein (UI) or a balanced (WI) diet were obtained 4 months after infection. Titers of IgG1, IgG2a, IgG2b or IgG3 anti-SEA antibodies were determined by ELISA. Data represent the mean ± SD of 7-8 mice per group. * $P<0.005$.

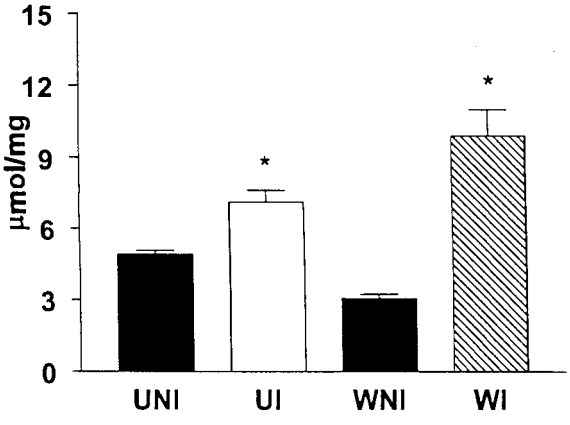

Figure 3: Quantification of collagen in livers of well-nourished and undernourished mice. Undernourished infected (UI) or noninfected (UNI) mice and well-nourished infected (WI) or noninfected (WNI) mice were sacrificed 4 months after *S. mansoni* infection. Collagen content in livers of mice from the different experimental groups was determined by measurement of hydroxyproline in liver samples as described in materials and methods. Results represent the mean ± SD of 7-8 mice per group. * $P<0.005$.

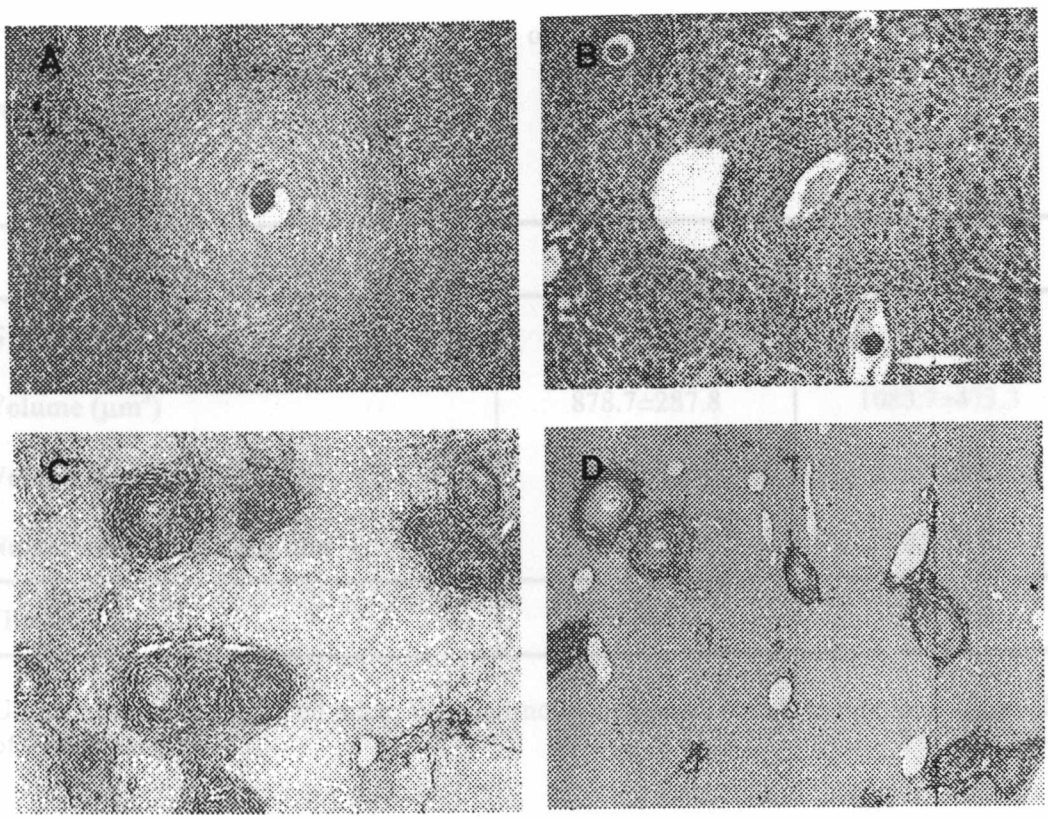

Figure 4: Periovular schistosomal granulomas in liver sections of mice 4 months after *S. mansoni*-infection. (A, C) Liver sections of well-nourished mice showing several ganulomas, with marked inflammatory response and dense collagen deposition. (B, D) Liver sections of undernourished mice showing small, perovular granulomas sparsely distributed throughout the hepatic parenchyma. A and B: H & E, 200 x; C and D: picrosirius red, 100 x.

Table 1. Morphometric study in livers of undernourished and well-nourished *S. mansoni*-infected mice.

	UI	WI
Granuloma		
Volume (μm^3)	878.7±287.8	1083.7±473.3
Volume density	187.0±35.1	197.9±30.5
Numerical density (nm/mm^{-4})	39.3±14.8	28.3±5.0
Fibrosis (%)	20.5±7.1	27.8±9.6

UI: undernourished infected mice; WI: well-nourished mice. Numbers represent means±SD of 9 mice per group.

Table 2. Cytokine production by spleen cells in undernourished and well-nourished *S. mansoni*-infected and non-infected mice.

	Medium		Con A		SEA	
Cytokines	**UI**	**WI**	**UI**	**WI**	**UI**	**WI**
IFN-γ	ND	22	102±107	72±58	ND	ND
IL-5	ND	ND	390±180	304±157	30±25	40±27

UI: undernourished infected mice; WI: well-nourished mice. Numbers represent means±SD of individual cultures of 7-8 mice per group (pg/ml)