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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Imunologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do Título de Doutor em Ciências.

Orientador: Prof. Dr. Alfredo Miranda de Goes

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ATA DA DEFESA DA TESE DE DOUTORADO DE CRISTINE HIRSCH MONTEIRO
REGISTRO 263

Aos vinte e hum dias do mês de março de 1997, às 10:00 horas, reuniu-se no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais a Comissão Examinadora da tese, indicada pelo Colegiado do Curso em 19 de dezembro de 1996, para julgar, em exame final, o trabalho intitulado "Participação de Antígenos do Schistosoma mansoni na Indução de Imunidade Protetora e na Modulação da Reação Granulomatosa" requisito final para a obtenção do grau de Doutor em Ciências: Imunologia. Abrindo a sessão o Presidente da Comissão, Prof. Alfredo Miranda de Góes da Universidade Federal de Minas Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra a candidata para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: - Prof. Dr. Franklin David Rumjanek da Universidade Federal do Rio de Janeiro, aprovada; Dra. Miriam Tendler da Fundação Oswaldo Cruz do Rio de Janeiro, aprovada; Profª Drª Maria de Fátima Martins Horta da UFMG, aprovada; - Prof. Dr. Carlos Alberto Pereira Tavares da UFMG, aprovada; Professor Dr. Alfredo Miranda de Góes da UFMG, aprovada. Pelas indicações a candidata foi considerada APROVADA. O resultado final foi comunicado publicamente a candidata pelo Presidente da Comissão. Nada mais havendo a tratar o Presidente da Comissão encerrou a reunião e lavrou a presente ATA que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 21 de março de 1997.

Drª Miriam Tendler - Fundação Oswaldo Cruz-RJ

Prof. Dr. Franklin David Rumjanek-UFRJ

Prof. Paulo Sérgio Lacerda Bueno
Coordenador do Curso de Pós-Graduação em
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Profª Drª Maria de Fátima Martins Horta-UFMG

Prof. Dr. Carlos Alberto Pereira Tavares-UFMG

Prof. Dr. Alfredo Miranda de Góes-UFMG

“Àqueles que sempre acreditaram que as coisas só podem vir a acontecer por nossos próprios esforços.”

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SUMÁRIO

LISTAS

ABREVIATURAS	Significado
Ac	Anticorpo(s)
AcM	Anticorpo(s) monoclonal(is)
AcMH	Anticorpo(s) monoclonal(is) humano(s)
Adj	Adjuvante
Ag	Antígeno(s)
AP	Antígeno(s) purificado(s)
BALB/c	Linhagem consanguínea de camundongo
C57bl/6	Linhagem consanguínea de camundongo
CaBP	Proteína Ligante de Cálcio
CAP	Preparação antigênica solúvel de cercárias
CD	Marcador glicoproteico diferencial de leucócitos ("cluster of differentiation")
cDNA	DNA complementar ao mRNA
DNA	Ácido desoxirribonucleico
DNArec	DNA recombinante
ESPG	Gene da proteína da casca do ovo do *S. mansoni*
ELISA	Ensaio imunoenzimático ligado à enzima
FPLC	Cromatografia líquida de rápida eluição de proteínas
fps	Ensaio de hipersensibilidade tardia no coxim plantar do animal experimental ("footpad swelling")
gp	Glicoproteína
GPI	Glicosil-fosfatidil-inusitol
GST	Glutationa S-transferase
G3PDH	Gliceraldeído 3-fosfato desidrogenase
HE	Hematoxilina-eosina
HGPRTase	Hipoxantina-guanidinorribosil transferase
HMGCoA	Hidroximetil-glutaril Co-enzima A
HPLC	Cromatografia líquida de alta performance
hsp	Proteína de choque térmico ("heat shock protein")
IC	Imunocomplexos (complexos Ag-Ac)
IFN	Inferferon
IL	Interleucina
k	kDa
LB	Linfócito(s) B

... continua...

... continuação:

ABREVIATURAS	Significado
LT	Linfócito(s) T
LTh	Linfócito(s) T auxiliares ("helper")
M?	Macrófago(s)
MNSP	Mononucleares dos sangue periférico
mRNA	RNA mensageiro
NK	Célula "natural killer" (exterminadora natural)
p	Antígeno proteico
p.c.	Percutâneo
PCI	Paciente esquistossomótico crônico-intestinal
PDI	Proteína dissulfeto isomerase
PM	Peso molecular
PIII	Fração aniônica obtida de SWAP em cromatografia
PIII/Adj	Imunização utilizando PIII na presença de adjuvantes
PR	Produto recombinante
pzq	Praziquantel
RNA	Ácido ribonucleico
SE	Extrato salino de produtos de excreção e secreção o verme adulto
SEA	Preparação antigênica solúvel de ovo
Sm	Designação genérica para Ag do *Schistosoma mansoni*
s.c.	Subcutâneo
SW	Sobrenadante de cultura de verme adulto
SWAP	Preparação antigênica solúvel de verme adulto
TA	Transferência adotiva de células
Teg	Ag do tegumento
TNF	Fator de Necrose Tumoral
TP	Transferência passiva
TPI	Triose fosfato isomerase
WEHL	Linhagem consanguínea de camundongos

RESUMO

Visando identificar componentes do ovo e do verme adulto do *Schistosoma mansoni* envolvidos na indução de imunidade protetora e na modulação da reação granulomatosa aos ovos deste helminto, empregamos técnicas de purificação e fracionamento partindo de extratos antigênicos solúveis destes estágios do parasito. Componentes da preparação antigênica solúvel de ovo (SEA) do *S. mansoni* reconhecidos por anticorpos monoclonais humanos (AcMH) provenientes de pacientes esquistossomóticos forma crônico-intestinal (PCI) foram obtidos em cromatografia de afinidade e foram bioquimicamente caracterizados quanto ao peso molecular e a capacidade destas frações em induzir proteção. Frações aniônicas obtidas a partir de preparação antigênica solúvel de verme adulto (SWAP) do *S. mansoni* foram obtidas em cromatografia de troca iônica usando coluna de Q-Sefarose acoplada ao sistema de FPLC. A reatividade imunológica humoral e celular induzida por este(s) Ag/frações frente a soro e células humanas e de animais de laboratório foi avaliada em ensaios de ELISA, proliferação celular e de formação de granuloma *in vitro*. Devido a sua reatividade *in vitro* com células mononucleares do sangue periférico (MNSP) de pacientes esquistossomóticos forma crônico-intestinal (PCI), a fração PIII, dentre as demais frações de SWAP, foi selecionada para ser utilizada nos experimentos de indução de proteção e modulação do granuloma *in vivo*. Camundongos BALB/c e C57bl/6 foram então imunizados com Ag purificados de SEA usando AcMH ou com PIII proveniente de SWAP. Em ambos os casos, foram obtidos graus significativos de proteção, próximos de 50%, avaliada através da recuperação de vermes adultos do sistema porta. Os granulomas dos animais imunizados com PIII foram comparados quanto à área do granuloma. Por fim, foi feita a seleção de clones codificadores de proteínas recombinantes em biblioteca de cDNA de verme adulto do *S. mansoni*, reativos com soro de coelho imunizado com PIII, buscando identificar os componentes antigênicos presentes nesta fração.

Palavras Chave: esquistossomose, granuloma, imunidade ativa, imunidade celular, imunidade humoral

ABSTRACT

To identify components of the egg and adult worm *Schistosoma mansoni* involved in the induction of protective immunity and modulation of granulomatous reaction to eggs, we employ techniques of fractionation and purification from soluble antigen extracts of these stages of the parasite. Components of soluble egg antigen preparation (SEA) of *S. mansoni* recognized by human monoclonal antibodies (AcMH) from schistosomiasis chronic intestinal patients (PCI) were obtained in affinity chromatography and were biochemically characterized with respect to molecular weight and the ability of these fractions to induce protection. Anionic fractions obtained from soluble antigen adult worm preparation (SWAP) of *S. mansoni* were obtained from anion exchange chromatography using Q-Sepharose column attached to the FPLC system. The humoral and cellular immune reactivity induced by this Ag/fractions against serum and human cells and experimental animals was evaluated in ELISA assays, cell proliferation and granuloma formation *in vitro*. Due to its *in vitro* reactivity with peripheral blood mononuclear cells (MNSP) from schistosomiasis patients chronic intestinal (PCI), the PIII fraction, among other fractions of SWAP, was selected to be used in experiments to induce protection and modulation the *in vivo* granulomatous reaction. BALB/c and C57bl/6 mice were immunized with purified Ag from SEA using AcMH or PIII from SWAP. In both cases, we obtained significant degrees of protection, close to 50%, as measured by recovery of adult worms in portal system. The granulomatous reaction of animals immunized with PIII was compared for the area of the granuloma. Finally, there was the scanning of clones encoding recombinant proteins in cDNA library of the adult worm *S. mansoni*, reactive rabbit serum immunized with PIII, seeking to identify the antigenic components present in this fraction.

Keywords: Schistosomiasis, granuloma, active immunity, cellular immunity, humoral immunity

1. INTRODUÇÃO

1.1. Uma visão da esquistossomose

A esquistossomose mansônica é uma endemia que afeta milhões de pessoas no Brasil sendo um dos problemas de saúde pública nacional. O helminto responsável por esta parasitose, *Schistosoma mansoni*, apresenta vários estágios evolutivos durante a fase de seu ciclo que se desenvolve no ser humano. Componentes das cercárias, estágio infectante, induzem formação de anticorpos (Ac) reativos a epitopos glicídicos que são liberados durante a transformação para esquistossômulo, verme jovem (CAPRON *et al.*, 1987). Estes Ac *in vitro* podem mediar uma reação de citotoxicidade ao verme, dependente de complemento ou células (eosinófilos, macrófagos ou plaquetas), de acordo com o seu isótipo (GRZYCH *et al.*, 1982; CAPRON *et al.*, 1984; HAGAN, 1992).

Durante a migração dos esquistossômulo, grande quantidade de antígenos também é liberada no corpo do hospedeiro gerando reatividade humoral e celular (SHER; Moser, 1981; OMER-ALI *et al.*, 1986; MOUNTFORD *et al*, 1995) que, em geral, são incapazes de causar dano ao próprio verme jovem ou mesmo ao verme adulto, pois os perfil antigênicos destas formas se mostram modificados quando se compara com o perfil do esquistossômulo que gerou esta reatividade. Esta resposta, entretanto, é responsável pela resistência adquirida contra novas reinfecções (KO; HARN, 1987; AITKEN *et al.*, 1988).

Os vermes adultos podem habitar um mesmo indivíduo por muitos anos (VIGNALI *et al.*, 1989) e, durante este período, o desenvolvimento de novos esquistossômulo é dificultado pela resposta imunológica induzida pelos vermes adultos já instalados (SMITHERS; TERRY, 1965 e 1967). Esta atividade imunológica é chamada de imunidade concomitante e afeta apenas os jovens esquistossômulo, não sendo eficaz no combate ao s vermes adultos, que permanecem intactos, apesar dos altos títulos de Ac e da reatividade celular dirigidos contra seus componentes (SMITHERS, 1972; SHER *et al.*, 1974; SMITHERS; TERRY, 1976; LEWIS; STRAND, 1991). Como mecanismos de escape ao ataque imunológico, o parasito usa a renovação constante de seu tegumento (TAVARES; GAZZINELLI, 1978; SIMPSON *et al.*, 1984), a síntese de moléculas semelhantes às do hospedeiro (CLEGG *et al.*, 1971; DAMIAN *et al.*, 1973; SHER *et al.*, 1978; DAMIAN *et*

al., 1984). Estas estratégias evitam a fixação do sistema complemento e fornecem uma camuflagem ao verme diante do sistema imunológico do hospedeiro (CLEGG *et al.*, 1971; DAMIAN *et al.*, 1973; SHER *et al.*, 1978; HARN *et al.*, 1985a; RAMALHO-PINTO, 1987).

A simples presença do verme não gera danos graves ao hospedeiro. Por outro lado, os ovos retidos, principalmente no fígado e intestinos, são responsáveis diretos pela patologia da esquistossomose (PHILLIPS; COLLEY, 1978; VON LICHTENBERG, 1987; BOROS, 1989), desencadeando uma resposta inflamatória granulomatosa (WARREN, *et al.*, 1967; BOROS, WARREN, 1970; HANG *et al.*, 1974). O granuloma gerado em torno dos ovos é composto por tipos celulares como macrófagos (Mϕ), células gigantes, fibroblastos, eosinófilos e linfócitos T e B (LT e LB) (MOORE *et al.*, 1977; LAMMIE *et al.*, 1986a; STADECKER, 1994). Na tentativa isolar e destruir o foco antigênico, esta resposta termina por gerar fibrose (LAMMIE *et al.*, 1986b; WYLER, 1992), com prejuízo da função original do tecido.

O número e a localização pericapilar dos granulomas fibrosados induzem uma congestão circulatória, que pode levar à hepatomegalia e/ou esplenomegalia, além de varizes esofagianas em casos mais graves (WARREN, 1982; BOROS, 1989). Entretanto, grande parte da população residente em áreas endêmicas não apresenta sintomatologia grave e convive com a infecção sem desenvolver complicações (WARREN, 1973; PRATA, 1978). Estes pacientes, chamados crônico-intestinais (PCI), apresentam granulomas diminuídos, quando comparados àqueles presentes em pacientes com formas graves da doença: fase aguda sintomática e crônica com complicações hepatoesplênicas (PRATA, 1978; BOROS, 1989).

O estudo da reatividade granulomatosa vem sendo feito usando modelos experimentais (ANDRADE; WARREN, 1964; COLLEY, 1976) e/ou ensaios *in vitro* (CHENSUE *et al.*, 1992). Análise de material histológico proveniente de infecções experimentais (CARTES; COLLEY, 1979; DAMIAN *et al.*, 1984; BOULANGER *et al.*, 1991; DUNNE *et al.*, 1991), ensaios de granuloma pulmonar (LUSTIGMAN *et al.*, 1985; LUKACS; BOROS, 1991a), assim como usando células de animais experimentais (CHESUE *et al.*, 1981) ou de humanos (CAMUS *et al.*, 1977; DOUGHTY; PHILLIPS, 1982; DOUGHTY *et al.*, 1987; GOES *et al.*, 1991; SILVA-TEIXEIRA *et al.*, 1996),

permitiram caracterizar a importância de diferentes tipos celulares e dos fatores solúveis por eles secretados na formação e modulação do granuloma.

As várias formas da doença são caracterizadas através de exames clínicos e o diagnóstico específico da infecção é feito através de exame parasitológico de fezes. Vários estudos vêm propondo o uso de antígenos (Ag) do parasito e outras proteínas em técnicas sorológicas para auxiliar no diagnóstico laboratorial (McLAREN *et al.*, 1981; CHAPPEL *et al.*, 1989; MANSOUR *et al.*, 1989; ALVES-BRITO *et al.*, 1992; RABELLO *et al.*, 1993), mas o sucesso não tem sido obtido devido à reatividade cruzada entre as diferentes espécies de *Schistosoma* e com outros vermes relacionados (MOSER *et al.*, 1990).

Antígenos do parasito também têm sido alvo de pesquisas para indução de proteção (PHILLIPS; COLLEY, 1978; MOTT, 1987; CAPRON *et al.*, 1987; BUTTERWORTH, 1992). Esta expectativa se baseia nas observações de que a infecção experimental (STIREWALT, 1953; HUNTER *et al.*, 1962; DEAN *et al.*, 1978a e b; BICKLE *et al.*, 1979), assim como a exposição persistente às cercárias em áreas endêmicas, protegem os indivíduos contra reinfecções (CLARK, 1966; BUTTERWORTH; TAYLOR, 1982; BUTTERWORTH; HAGAN, 1987; HAGAN, 1992).

Tanto para a sorodiagnose quanto para os estudos de indução de proteção, faz-se necessário o isolamento e a caracterização de Ag próprios do *S. mansoni* (SMITH; CLEGG, 1985; SIMPSON; CIOLI, 1987; MOSER *et al.*, 1990).

O perfil de reatividade imunológica dirigida aos Ag do parasito que se desenvolve com a infecção tem sido relacionado ao estabelecimento dos mecanismos que levam ao controle do tamanho do granuloma (OTTENSEN *et al.*, 1978; OTTENSEN, 1979; ELLNER, 1980; OTTENSEN; POINDEXTER, 1980; COLLEY, 1981a e b; COLEY *et al.*, 1986; GAZZINELLI *et al.*, 1987; REZENDE *et al.*, 1993 e 1997) e a indução de imunidade protetora (STIREWALT, 1953; CLARK, 1966; BUTTERWORTH; TAYLOR, 1982; HARN *et al.*, 1984; HAZDAI *et al.*, 1985; SMITH; CLEGG, 1985; SHER *et al.*, 1986; CAPRON *et al.*, 1987; GREGOIRE *et al.*, 1987; KING *et al.*, 1987; BUTTERWORTH, 1992; HAGAN, 1992; SOISSON *et al.*, 1992). Em nosso trabalho, estas duas abordagens são enfocadas.

1.2. Regulação da resposta granulomatosa dirigida contra os ovos do parasito

Estudos usando infecção experimental de camundongos têm mostrado que os granulomas hepáticos de animais com oito semanas de infecção são maiores que daqueles com 32 semanas (ANDRADE; WARREN, 1964; PHILLIPS; COLLEY, 1978). Isto mostra a diminuição da reação granulomatosa ao longo da cronificação da doença (COLLEY, 1981b e 1987). Na esquistossomose humana, por sua vez, verifica-se que, na maior parte dos indivíduos infectados em áreas endêmicas, a cronificação da infecção leva ao estabelecimento de uma forma clínica assintomática, camada intestinal (PRATA, 1978), acompanhada da supressão da reação granulomatosa (COLLEY, 1981b e 1987).

Diferentes fatores, tanto do hospedeiro, quanto do parasito, têm sido associados ao estabelecimento da modulação da resposta inflamatória dirigida aos ovos do parasito (*TAB.* 1).

TABELA 1: Mecanismos moduladores da resposta granulomatosa aos ovos do parasito

Modulador	Evidências	Referências
Padrões genéticos	HLA	ABDEL-SALAM *et al.*, 1979 E 1986
	"Raça"	TAVARES-NETO; PRATA, 1990
	Tipo sanguíneo	LIMA-PEREIRA *et al.*, 1979
Ag do parasito	Vermes x lesão	ELLNER *et al.*, 1981; BOULANGER *et al.*, 1991
	gp do ovo x lesão	CARTER; COLLEY, 1981; LUSTIGMAN *et al.*, 1985
IC	Inibindo Mϕ	GOES *et al.*, 1991; REZENDE *et al.*, 1993
	Prostaglandinas	GOES *et al.*, 1994
Macrófagos	Fatores solúveis	OTTENSEN, 1979; LUKACS *et al.*, 1995
	Apresentação de Ag	TODD *et al.*, 1979; MENSON; WILSON, 1989
	Granuloma *in vivo*	CHENSUE *et al.*, 1995b; LUKACS *et al.*, 1995
	Granuloma *in vitro*	SILVA-TEIXEIRA *et al.*, 1993 E 1996
LT	Camundongos atímicos	DOENHOFF *et al.*, 1981
	Transferência adotiva	COLLEY, 1976
	Reatividade *in vitro*	COLLEY *et al.*, 1977; BAHIA-OLIVEIRA *et al.*, 1992
	CD8+	CHENSUE *et al.*, 1981; MATHEW; BOROS, 1986
	CD4+	DOUGHTY *et al.*, 1987; CONTIGLI *et al.*, 1994
Citocinas	Th1 x Th2	CHER; MOSMANN, 1987
	Diminuição/Aumento da reação granulomatosa	CHIKUNGUWO *et al.*, 1991; GRZYCH *et al.*, 1991; LUKACS; BOROS, 1992; WYNN; CHEEVER, 1995; KING *et al.*, 1996; SILVA-TEIXEIRA; DOUGHTY; GOES, 1996
Interações	Linfócitos T	PHILLPS *et al.*, 1980; DOUGHTY *et al.*, 1989
	Anticorpos	PARRA, *et al.*, BOSSHARDT *et al.*, 1996

Antígenos de vários estágios do parasito são capazes de ativar LT citotóxicos do hospedeiro (MATHEW; BOROS, 1986). Componentes antigênicos do ovo (CARTER; COLLEY, 1979; LUSTGMAN *et al.*, 1985), do esquistossômulo (DAMIAN *et al.*, 1984) e de vermes adultos (ELLNER *et al.*, 1981; BOULANGER *et al.*, 1991) exacerbam ou suprimem a formação de granuloma dependendo do ensaio experimental utilizado. Ag de vários estágios do parasito são capazes de induzir diferentes perfis de secreção de citocinas por LT auxiliares (MOUNTFORD *et al.*, 1995; CHENSUE *et al.*, 1995a).

Da mesma forma, fatores genéticos do hospedeiro, como componentes raciais (TAVARES-NETO; PRATA, 1990), genes do complexo principal de histocompatibilidade – HLA (ABDEL-SALAM *et al.*, 1979 e 1986) e tipo sanguíneo (LIMA-PEREIRA *et al.*, 1979), também têm sido associados à modulação da reação granulomatosa.

Dentre os componentes do infiltrado celular, LT e Mϕ têm papel reconhecido na modulação do granuloma. Macrófagos têm sido associados á modulação da resposta através da liberação de fatores solúveis (OTTENSEN, 1979; TODD *et al.*, 1979). Estes fatores funcionam como quimiotáticos para outras células, as quais irão colaborar para a eliminação do foco antigênico (OTTENSEN, 1979; LUKACS *et al.*, 1994; CHENSUE *et al.*, 1995; LUKACS *et al.*, 1995). Macrófagos também são responsáveis pela apresentação de Ag na esquistossomose (TODD *et al..*, 1979; MENSON; WILSON, 1989) que irá mobilizar linfócitos a participarem do processo. Com a diferenciação dos Mϕ em células gigantes, frequentemente evidenciadas em cortes histológicos de granulomas (MOORE *et al.*, 1977), os padrões de secreção de fatores solúveis e citocinas (JANSON *et al.*, 1990; CHESUE *et al.*, 1989; RUPPE; PETERS, 1991), de apresentação de Ag (SILVA-TEIXEIRA *et al.*, 1993) e mesmo o perfil de marcadores de membrana (ANDEERSEN *et al.*, 1987; BECKER *et al.*, 1987; WAHL *et al.*, 1987; GEISSLER *et al.*, 1989; BLANCHARD; DJEU, 1991; SILVA-TEIXEIRA *et al.*, 1996) se apresentam bastante modificados.

REZENDE *et al.* (1993) demonstraram que células do sangue periférico de pacientes esquistossomóticos tiveram sua capacidade de formação de granulomas *in vitro* suprimida quando previamente tratadas como imunocomplexos. Este papel foi atribuído à prostaglandinas liberadas pelos Mϕ (GOES *et al.*, 1994).

Já a participação dos LT na modulação do granuloma vem sendo estudada a partir de infecções experimentais em camundongos atímicos ou desprovidos de LT, quando

Phillips *et al.* (1980) e Doenhoff *et al.* (1981) observaram que os granulomas não são formados de maneira eficiente nestes animais, isto é, o infiltrado celular se encontra desorganizado. Também foram evidenciadas lesões disseminadas em diferentes órgãos, devido à não eliminação e/ou contenção do foco antigênico (DOENHOFF *et al.*, 1981; VON LICHTENBERG, 1987). A importância de LT no processo de formação e modulação do granuloma é reforçada por ensaios de transferência adotiva de células, mostrando que o padrão de reatividade granulomatosa de animais doadores pode ser induzido em animais receptores deste tipo de célula (COLLEY, 1976; COLLEY *et al.*, 1979).

Experimentos *in vitro* com células humanas também contribuíram para o estabelecimento do papel dos LT (DOUGHTY *et al.*, 1987). A reatividade diferencial de LT provenientes de pacientes portadores de variadas formas clínicas, portanto de diferentes formas da patologia, frente a Ag do parasito e mesmo a mitógenos, sugeriu o papel modulador deste tipo de célula (COLLEY *et al.*, 1977; GAZZINELLI *et al.*, 1987; BAHIA-OLIVEIRA, *et al.*, 1992).

Citocinas produzidas por LT CD4+, LT auxiliares (LTh), vêm demonstrando desempenhar importante papel imunomodulador em infecções por vírus, fungos, protozoários e helmintos (SCOTT; KAUFMAN, 1991; WYNN; CHEEVER, 1995). LTh vêm sendo caracterizados a partir de culturas de células humanas ou provenientes de animais experimentais e diferentes padrões de secreção de citocinas foram identificados (CHER; MOSMANN, 1987; CONTIGLI *et al.*, 1994; DEL PRETE; ROMAGNANI, 1994). Estes perfis de secreção de citocinas têm sido usados para caracterizar subtipos de LTh, já que marcadores de superfície capazes de distinguir cada subtipo ainda não foram descritos (SCOTT; KAUFMAN, 1991).

Clones de LTh tipo 1 (LTh1) secretam IFN-γ, IL-2 e TNF-β (linfotoxina), e se relacionam diretamente à ativação de uma resposta imunológica celular (CHER; MOSMANN, 1987; CHIKUNGUWO, 1991; DEL PRETE, 1992; DEL PRETE; ROMAGNANI, 1994) mediada pela apresentação de Ag preferencialmente por Mϕ (GAJEWSKI *et al.*, 1991). Por outro lado, clones de LTH tipo 2 (LTh2) secretam IL-4, IL-5, IL-6 e IL-13, tendo seu papel voltado para a ativação de resposta humoral (CHER; MOSMANN, 1987; MINTY *et al.*, 1993) e dependem preferencialmente de LB como células apresentadoras de Ag (GAJEWSKI *et al.*, 1991).

A IL-10, citocina supressora da atividade de Mϕ, portanto, inibidora da secreção de citocinas Th1, é produzida por vários tipos celulares, incluindo Mϕ, LB e LTh. Em camundongos é produzida apenas por LTh2 (HOWARD *et al.*, 1992; FLORES-VILLANUEVA *et al.*, 1993), entretanto, os dois tipos de clones humanos, LTh1 e LTh2, produzem esta citocina (DEL PRETE *et al.*, 1993).

Importante citocina para a diferenciação dos perfis de secreção de citocinas pelos LTh é a IL-12 que é, por sua vez, produzida apenas por monócitos/macrófagos e induz a produção de e secreção de IFN-γ por células NK. A principal ação desta citocina é a ativação do perfil de secreção de citocinas Th1 e inibição de clones de LTh2 (MANETTI *et al.*, 1993).

No caso da esquistossomose experimental, tem disso demonstrado que, antes da deposição dos ovos, ou seja, nas primeiras quatro semanas pós-infecção, o padrão de secreção de citocinas é Th1 (CHIKUNGUWO *et al.,* 1991; LUKACS; BOROS, 1992). Entretanto, com o início da oviposição, ocorre a indução da secreção de citocinas Th2, como IL-4 e IL-5 (GRZYCH *et al.*, 1991) e IL-10 (FLORES-VILLANUEVA *et al*, 1993). Esta diferenciação parece ser induzida por IL-4, cuja fonte seriam os eosinófilos do próprio granuloma (SABIN *et al.*, 1995). Na fase crônica da infecção, os níveis de citocinas são bem menores que na fase aguda (HENDERSON *et al.*, 1992). Experimentos de neutralização (camundongos "knockout" ou o uso de Ac específicos para uma citocina) e/ou reconstituição com citocinas exógenas (*in vivo* ou *in vitro*) vêm esclarecendo o papel das diferentes citocinas no desenvolvimento e na modulação do granuloma.

A IL-4 parece ser indutora da transformação do granuloma, pois a neutralização *in vivo* desta citocina induz a diminuição do granuloma (YAMASHITA; BOROS, 1992), assim como da deposição de colágeno (CHEEVER *et al.*, 1994 e 1995), a diminuição do granuloma é acompanhada pela diminuição da síntese de mRNA para IL-4 e IL-5 *in vivo* (WYNN *et al.*, 1993).

Citocina com importante papel na proliferação e diferenciação de eosinófilos, a IL-5 demonstrou ser responsável pela eosinofilia associada ao granuloma na fase aguda da infecção, enquanto que, na fase crônica, a eosinofilia parece estar associada a fatores secretados pelas células aderentes (EL-CHEIKH; BOROJEWIC, 1990; EL-CHEIKH; DUTRA; BOROJEWIC, 1991).

Em contrapartida, a IL-12 seria a citocina moduladora negativa da reatividade granulomatosa, uma vez que experimentos de neutralização demonstram aumento do granuloma à custa de citocinas Th2 e outros onde se fez a adição de IL-12 houve a supressão do granuloma acompanhada de diminuição das citocinas Th2 (WYNN *et al.*, 1994) e pela diminuição da fibrose (WYNN *et al.*, 1995).

O IFN-γ, considerado uma citocina anti-inflamatória (LAMMIE *et al.*, 1986a e b), tem um papel duplo quanto ao desenvolvimento e à modulação do granuloma, pois sua neutralização na fase aguda da infecção aumenta a lesão (LUKACS; BOROS, 1991a; CHENSUE *et al.*, 1992; CHENSUE *et al.*, 1994; WYNN *et al.*, 1994; REZENDE *et al.*, 1995). Além disso, a adição de IFN-γ exógeno diminuir tanto o granuloma pulmonar quanto o hepático (LUKACS; BOROS, 1993).

A IL-10 já é reconhecida como uma citocina tarda na formação do granuloma e atua diminuindo a expressão de moléculas apresentadoras de Ag e coestimulatórias, principalmente em Mϕ, diminuindo a apresentação de Ag para LTh (CORRY *et al.*, 1994). O efeito desta citocina em diferentes ensaios tem mostrado resultados controversos. Em alguns experimentos, a neutralização da IL-10 na fase aguda da infecção não tem efeito observável sobre o granuloma (WYNN *et al.*, 1994), enquanto que na fase crônica aumenta a supressão do granuloma dirigida por LT específicos para Ag solúvel de ovos (SHER *et al.*, 1991; FLORES-VILLANUEVA *et al.*, 1996). Esta supressão é acompanhada pela diminuição da produção de IFN- γ (SHER *et al.*, 1991). Entretanto, em experimentos de adição, esta citocina induz, na fase aguda, uma sensível diminuição de citocinas Th2 e pouco efeito na produção de citocinas Th1, enquanto que na fase crônica, produz uma marcante redução na secreção de ambos os tipos de citocinas. Parece que LTh1 são menos sensíveis à IL-10 durante a fase de formação do granuloma (CORRY *et al.*, 1994). Por outro lado, em outros ensaios, apenas uma proteína de fusão IL-10/Fc, com meia vida mais longa, foi capaz de diminuir o tamanho do granuloma hepático e este resultado foi acompanhado pela supressão de citocinas Th2 (FLORES-VILLANUEVA *et al.*, 1996).

Outra citocina de grande importância para a ativação e proliferação de linfócitos é a IL-2, que age indiferentemente tanto em LTh1 como em LTh2. A neutralização desta citocina, na fase aguda diminui tanto o granuloma quanto a fibrose, o que é acompanhado pela diminuição na eosinofilia tecidual e da secreção de IL-5 (CHEEVER *et al.*, 1992). A adição de IL-2 na fase aguda aumenta o granuloma, provavelmente a custa da estimulação

de LTh2 (CHIKUNGUWO *et al.,* 1991), e, na fase crônica, aumenta a supressão do granuloma (MATHEW *et al.*, 1990), aumentando a secreção de citocinas Th1 (CHENSUE *et al.*, 1992). Estas afirmações são confirmadas por experimentos de inativação de células portadoras de receptor de IL-2 de alta afinidade, onde foi obtida a supressão da formação do granuloma e da deposição de colágeno no fígado de camundongos infectados (RAMADAN *et al.*, 1995).

Outros fatores solúveis, produzidos por várias células acessórias, também têm mostrado importante papel na formação do infiltrado inflamatório da reação granulomatosa. O TNF-α, ao contrário da IL-10, induz a expressão de moléculas de adesão em leucócitos, quando neutralizado induz a redução do tamanho do granuloma (LUKACS *et al.*, 1994; CHENSUE *et al.*, 1995a). Experimentos *in vitro* confirmam estes achados na medida em que a diminuição da proliferação celular e da formação do granuloma Ag-específicos induzida por imunocomplexos é acompanhada pela diminuição desta citocina e do aumento de IL-10 (REZENDE *et al.*, 1997). MIP-1α ("macrophage inflamatory protein 1 alpha") é quimiocina para eosinófilos e a estimulação com SEA inalável induz aumento da expressão deste fator no pulmão de camundongos (LUKACS *et al.*, 1995). A neutralização do fator MCP ("monocyte chemotactic protein") diminui a área do granuloma em 40% e sua produção é regulada por LT (CHENSUE *et al.*, 1995b).

Diferentes padrões nas interações idiotípicas de Ac têm sido evidenciados em pacientes com as várias formas clínicas da doença (MONTESANO *et al.*, 1989). Além disso, interações idiotípicas entre Ac e/ou LB e LT têm se mostrado capazes de regular a formação *in vitro* do granuloma (DOUGHTY *et al.*, 1989; PARRA *et al.*, 1991). Também foi observado que a evolução do padrão de Ac anti-SEA e LT antidiotípicos está relacionada à cronificação da doença (BOSSHARDT *et al.*, 1996).

1.3. Indução de imunidade protetora

Imunidade adquirida com a infecção (STIREWALT, 1953; CLARK, 1966) sugere que Ag do parasito possam ser usados como medida profilática (CAPRON *et al.*, 1987). Como problema de saúde dos mais sérios nos países em desenvolvimento, inúmeros estudos (*TAB.* 2) têm sido desenvolvidos para se obter uma vacina para a esquistossomose.

TABELA 2: Antígenos do *Schistosoma mansoni* e indução de proteção:

Estágio evolutivo	Extrato ou Fração	DTH[1]	Proteção[2]	Referências
Ovos	SEA*	+ Pulmonar	X	LUSTIGMSN *et al.*, 1985
	Lectina	Inalterada	X	LUSTIGMSN *et al.*, 1985
		+ Hepática	X	CARTER; COLLEY, 1979
	AcM	X	TP 41%	HARN *et al.*, 1984
	Troca Catiônica	TP - Hepática	X	DUNNE *et al.*, 1991
	Eletroforese	+/- Pulmonar	X	LUKACS; BOROS, 1991a
Cercárias	Irradiadas	X	42%	RUPPEL *et al.*, 1990
		X	67%	DEAN, 1983
		X	51%	KANIYA *et al.*, 1993
		X	50%[3]	YOLE *et al.*, 1996
Verme jovem	Transformado**	- Hepática[3]	6%[3]	DAMIAN *et al.*, 1984
	AcM	X	AcM 54%	ZODDA; PHILLIPS, 1982
	AcM	X	AP(155k)42%, 42%[3] AP(53k) 31%	SMITH; CLEGG, 1985
	AcM	X	AP(28k) 40% AP(38k) 37%	HARN *et al.*, 1987
	AcM	fps e pulmonar inalterados	AcM 27% AP(68k) 66%	KING *et al.*, 1987
	AcM	X	TP 35-42%, 50%[6] AP(200k) 34%	HAZDAI *et al.*, 1985
	AcM	X	TP 32% e 41%	BICKLE *et al.*, 1986
Verme adulto	SWAP*	X	67%[3]	SHER *et al.*, 1986
	SE***	X	AP 90%[4]	TENDLER, 1987
	SW/pzq****	X	67%	FALLON; DOENHOFF, 1995
	Gel filtração	X	AP(97k) 60%	SHER *et al.*, 1986
	Cromatografia	X	AP(97k) 50%	PEARCE *et al.*, 1988
	AcM	X	TP 39%	GREGOIRE *et al.*, 1987
	DNArec	X	PR(GST) 60%[5]	BALLOUL *et al.*, 1991
	DNArec	PR – Hepática[3] TA – 30%	PR(GST) 38%[3]	BOULANGER *et al.*, 1991 PANCRE *et al.*, 1994a e b
	DNArec	X	PR(miosina) 75%	SOISSON *et al.*, 1992
	DNArec	X	PR(Sm14) 69%	TENDLER *et al.*, 1996

Legenda: 1. Resposta de hipersensibilidade tardia: granuloma hepático ou pulmonar, ou ensaio de "footpad swelling" (fps); (+) indica aumento e (-) indica diminuição; **2.** Ensaio de proteção (recuperação de vermes adultos) em camundongos, ou **3.** Macaco, ou **4.** Coelho, ou **5.** Rato. **6.** Morte *in vitro* de esquistossômulo.
* Extratos solúveis de ovo (SEA), cercária (CAP) e verme adulto (SWAP);
** Esquistossômulos mecanicamente transformados *in vitro* a partir de cercárias irradiadas;
*** Extrato salino de produtos de excreção ou secreção;
**** Imunização com sobrenadante de cultura de verme adulto associada ao tratamento com praziquantel
Siglas: **AcM** – anticorpo monoclonal; **TP** – transferência passiva; **TA** – transferência adotiva de células; **AP** – imunização com Ag purificado (peso molecular indicado entre parêntesis); **PR** – produto recombinante.

A vacina para esquistossomose deve ser capaz de proteger o indivíduo da infecção pelo *S. mansoni* ou pelo menos limitar o desenvolvimento das fêmeas, diminuindo assim a oviposição, e, por consequência, a patologia gerada pela formação dos granulomas em torno dos ovos (WHO, 1991; BERGQUIST, 1995a e b; CAPRON *et al.*, 1995).

Experimentalmente, tem sido demonstrado que o soro de animais infectados cronicamente é capaz de transferir proteção contra infecção desafio (MANGOLD; DEAN, 1986). Experimentos *in vitro* têm mostrado que anticorpos monoclonais (AcM), provenientes de baço de animais infectados ou imunizados, são capazes de causar dano ao esquistossômulo (RAMALHO-PINTO *et al.*, 1979), sendo necessária a presença de células e complemento (ANWAR *et al.*, 1979). A fim de avaliar os mecanismos desencadeados pela transferência de soro, vários pesquisadores partiram para a produção de AcM específicos para Ag do S. *mansoni* (HARN *et al.*, 1984; HAZDAI *et al.*, 1985; BICKLE *et al.*, 1986; GREGOIRE *et al.*, 1987; GOES *et al.*, 1989).

Ensaios de transferência passiva demonstraram que vários destes anticorpos fornecem proteção (HARN *et al.*, 1984; HAZDAI *et al.*, 1985; BICKLE *et al.*, 1986; GREGOIRE *et al.*, 1987) ou mediam citotoxicidade celular *in vitro* contra esquistossômulos (HAZDAI *et al.*, 1985). Estes estudos levaram inúmeros pesquisadores a utilizar Ac com atividade protetora para obter Ag protetores (CAPRON *et al.*, 1987; SIMPSON; CIOLI, 1987; COLLEY; COLLEY, 1989; WRIGHT *et al.*, 1991; PEARCE *et al.*, 1993).

À exceção de cercárias irradiadas, que fornecem graus relevantes de proteção (DEAN *et al.*, 1983; COHEN; EVELAND, 1988; KAMIYA *et al.*, 1993; YOLE *et al.*, 1996), extratos antigênicos de ovo (HARN *et al.*, 1984), de cercárias (HOROWITZ *et al.*, 1982), de esquistossômulos (HAZDAI *et al.*, 1985; SMITH; CLEGG, 1985; SHER *et al.*, 1986) e de vermes adultos (SHER *et al.*, 1986) promovem variados graus de proteção contra uma infecção desafio em animais experimentais permissivos, predominando baixos níveis. Além disso, coelhos (TENDLER, 1987), ratos (BALLOUL *et al.*, 1987) e macacos (SMITH; CLEGG, 1985; SHER *et al.*, 1986; BOULANGER *et al.*, 1991), animais naturalmente não permissivos à infecção pelo S. *mansoni* (SMITHERS; TERRY, 1965 e 1967), apresentam níveis de proteção mais significativos.

Fallon e Doenhoff (1995) associaram a imunização ao tratamento anti-helmíntico mostrando que, apesar de não apresentar efeito protetor isoladamente, a imunização de camundongos com o extrato de membrana do verme adulto (SW) aumenta em 67% a eficiência do praziquantel. Alguns Ag-alvo para Ac só ficam expostos na superfície do verme adulto após danos provocados pela droga esquistossomicida como, por exemplo, o

praziquantel, e uma resposta imunológica contra estes epitopos pode melhorar a eficácia do tratamento (BRINDLEY *et al.*, 1989).

Componentes antigênicos do esquistossômulo (ZODDA; PHILLIPS, 1982; HAZDAI *et al.*, 1985; SMITH; CLEGG, 1985; BICKLE *et al.*, 1986; HARN *et al.*, 1987) e do verme adulto (BALLOUL *et al.*, 1987; GREGOIRE *et al.*, 1987; TENDLER, 1987; SHER *et al.*, 1986; PEARCE *et al.*, 1988; SOISSON *et al.*, 1992) têm sido estudados com relação á proteção contra infecção, enquanto que os componentes dos ovos (CARTER; COLLEY, 1977; LUSTIGMAN *et al.*, 1985; DUNNE *et al.*, 1991; LUKACS; BOROS, 1991a) têm sido avaliados em função da indução da patologia. Vários pesquisadores sugerem que componentes do verme adulto podem ter papel na reatividade imunológica contra os ovos do parasito (ELLNER *et al.*, 1981; CAPRON, 1985; DOUGHTY; PHILLIPS, 1982a). Tanto ovos como vermes se localizam no microambiente da circulação portal, onde o verme libera constantemente Ag de sua superfície e produtos de excreção/secreção que podem exercer papel imunorregulador sob a reação granulomatosa (DOUGHTY; PHILLIPS, 1982a; TENDLER, 1987).

1.4. Isolamento e caracterização de Ag do parasito

Para avaliar com mais profundidade a base molecular de processos como a indução de imunidade protetora e da modulação da reação granulomatosa é de fundamental importância que componentes puros e definidos do parasito estejam disponíveis (KNIGHT *et al.*, 1986). Das técnicas de separação ou isolamento de Ag normalmente empregadas, o uso de Ac em ensaio de imunoprecipitação (HARN *et al.*, 1984; HAZDAI *et al.*, 1985; BICKLE *et al.*, 1986) ou colunas de afinidade (CARTER; COLLEY, 1979; HARN *et al.*, 1985a e b; LUSTIGMAN *et al.*, 1985; KING *et al.*, 1987) fornecem altos graus de pureza, entretanto com rendimentos muito baixos. Em geral, Ac com atividade protetora são dirigidos contra proteínas de membrana altamente glicosiladas, o que dificulta a caracterização dos Ag-alvo e o entendimento de seu mecanismo de ação (KNIGHT *et al.*, 1986; OMER-ALI *et al.*, 1986). Através de cromatografias de troca iônica em sistemas de alta resolução (FPLC ou HPLC) (PEARCE *et al.*, 1988; DUNNE *et al.*, 1991), assim como

a focalização isoelétrica (McLAREN *et al.*, 1981; HARN *et al.*, 1989) vários Ag também vêm sendo obtidos (*TAB.* 2).

Através de cromatografia de afinidade, usando AcM com atividade protetora, em ensaios de transferência passiva ou de morte *in vitro* de esquistossômulos (Ac letal), foram obtidos diferentes componentes que, em ensaios de imunização, apresentaram variados graus de proteção. Partindo do esquistossômulo, Smith e Clegg (1985) caracterizaram componentes com 53kDa e 155kDa e Hazdai *et al.* (1985) obtiveram um componente de 200kDa que induziram níveis de proteção em torno de 30% na infecção experimental em camundongos. Harn *et al.* (1987) purificaram um componente de 38kDa e outro de 28kDa (TPI – triose fosfoisomerase) e King *et al.* (1987) caracterizaram outro de 68kDa que também foram capazes de induzir proteção em modelos experimentais. Usando gel filtração, SHER *et al.* (1986) purificaram, a partir de verme adulto, um componente de 97kDa, identificado como sendo a paramiosina, capaz de induzir 60% de proteção em camundongos.

A tecnologia do DNA recombinante possibilita alto grau de pureza e rendimento bastante satisfatório, e o produto isolado é função direta do genoma de interesse (KNIGHT *et al.*, 1986). Deste modo, inúmeros componentes do parasito foram clonados por engenharia genética, visando obter Ag suficientemente isolados e em grande quantidade. Várias têm sido as estratégias utilizadas para a pesquisa inicial dos antígenos (*TAB.* 3a e 3b), como soro de animais experimentais resistentes à reinfecção (LANAR *et al.*, 1985; DALTON *et al.*, 1987; WRIGHT *et al.*, 1988; EL SHERBEINI *et al.*, 1991) ou por Ac de pacientes esquistossomóticos (STEIN; DAVID, 1986; SIMURDA *et al.*, 1988; GOUDOT-CROUZEL *et al.*, 1989; XU *et al.*, 1989). Foram usadas bibliotecas genômicas (CRAIG *et al.*, 1988; RAJKOWICK *et al.*, 1989; FINKEN *et al.*, 1994) ou de cDNA (KNIGHT *et al.*, 1989; BOULANGER *et al.*, 1991) de diferentes estágios do S. *mansoni*, construídas em diferentes vetores de clonagem ou de expressão. Usando sondas de oligonucleotídeos (CRAIG *et al.*, 1988; NEWPORT *et al.*, 1988; EL SHERBEINI *et al.*, 1991) ou Ac provenientes de animais experimentalmente imunizados ou infectados (LANAR *et al.*, 1985; DALTON *et al.*, 1987; KLINKERT *et al.*, 1987; WRIGHT *et al.*, 1988; KNIGHT *et al.*, 1989; EL SHERBEINI *et al.*, 1991; JEFFS *et al.*, 1991) ou de pacientes (STEIN; DAVID, 1986; HEDSTROM *et al.*, 1987 e 1988), têm sido isolados diferentes produtos recombinantes. O uso destes produtos em experimentos de imunização muito tem esclarecido sobre sua participação na imunidade contra o parasito.

Dos vários produtos recombinantes obtidos, a SM28-GST (BALLOUL *et al.*, 1987; BOULANGER *et al.*, 1991) e miosina (SOISSON *et al.*, 1992) apresentaram 60% (em rato) e 75% (em camundongo) de proteção, respectivamente. As GST do S. *mansoni*, Sm26 (TIU *et al.*, 1988; TROTTEN *et al.*, 1990) e Sm28 (BALLOUL *et al.*, 1987; BOULANGER *et al.*, 1991), já foram recomendadas pela Organização Mundial de Saúde como candidatas à vacina em seres humanos, assim como a paramiosina (SHER *et al.*, 1986) e a TPI (28k), caracterizada por Harn *et al.* (1987) e clonada por Schoemaker *et al.* (1992).

Outros Ag com variados pesos moleculares têm sido clonados a partir do DNA de vermes adultos e tecnologia do DNA recombinante: a proteína principal do ovo – ESPG (KOSTER *et al.*, 1988) e a Sm25 (WRIGHT *et al.*, 1988; OMER-ALI *et al.*, 1991). Na região de 36kDa temos a G3PDH (DESSEIN *et al.*, 1988), a hemoglobinase (KLINKERT *et al.*, 1987 e 1988), a superóxido dismutase (SIMURDA *et al.*, 1988) e a tropomiosina (XU *et al.*, 1989). Na faixa de 50-70kDa temos a PDI (FINKEN *et al.*, 1994) e uma hsp (HEDSTROM *et al.*, 1987 e 1988). Com algo PM foi clonada a miosina (NEWPORT *et al.*, 1988a; SOISSON *et al.*, 1992).

TABELA 3a: Ag do *S. mansoni* clonados por tecnologia do DNA recombinante

Estágio	Ag clonado	Característica da pesquisa		Referências
		Sonda	Relevância	
Ovo	p40	Ac coelho αSEA	Diagnóstico específico	CONDINGLEY *et al.*, 1983
	p40-2	Ac coelho αSEA	Homologia com hsp	CAO *et al.*, 1993
	Proteína ligadora de Ca++	Soro humano crônico	Reconhecido por soro humano	MOSER *et al.*, 1992
Esporocisto	Elastase	Nucleotídeo	Infecção	NEWPORT *et al.*, 1988b
Cercária	Creatinina quinase (74k)	Nucleotídeo	Clone estágio-específico	STEIN *et al.*, 1990
	Tropomiosina (49k)	Nucleotídeo	Ac paciente e camundongo vacinado	XU *et al.*, 1989
	TPI (28k)	Nucleotídeo	TP (AcM)	HARN *et al.*, 1985b e 1987 SHOEMAKER *et al.*, 1992
	CaBP	Nucleotídeo (= gene humano)	Não é expressa em esporocisto e adulto	RAM *et al.*, 1986
	Serinoprotease (28k)	Nucleotídeo	Ag secretado com ação sobre complemento	MARIKOVSKY et al., 1990 FISHELSON et al., 1992

Legendas: TP – transferência passiva de imunidade (soro ou Ac); **AcM** – anticorpo monoclonal; **Siglas** (ordem de aparição): **p** – proteína; **hsp** – "heat shock protein"; **TPI** – triose fosfoisomerase; **CaBP** – proteína ligadora de cálcio.

TABELA 3b: Ag do *S. mansoni* clonados por tecnologia do DNA recombinante a partir do verme adulto

Ag clonado	Característica da pesquisa		Referências
	Sonda	Relevância	
22,6k	Ac de paciente		STEIN; DAVIS, 1986
70k	Ac camundongo crônico	Ac de animal protegido	LANAR *et al.*, 1985
Paramiosina (97k)	Ac coelho α AP	Ac de paciente resistente	LANAR *et al.*, 1986
		AP protege camundongo	CORREIA-OLIVEIRA *et al.*, 1989 PEARCE *et al.*, 1986
Sm28 (GST) Peptídeos 190-211	Ac rato e coelho α AP	AP protege	BALLOUL *et al.*, 1987 BOULANGER *et al.*, 1991 TAYLOR *et al.*, 1988
	Ac rato e coelho α AP	Diminui fibrose	PANCRÉ *et al.*, 1994a e1994b
Proteases 31-32kDa (catepsina B e hemoglobinase)	Ac coelho α AP	Produto de secreção e excreção	KLINKERT *et al.*, 1987 e 1989 DAVIS *et al.*, 1987 FELLEISEN *et al.*, 1990
18k	Ac rato e coelho α AP	Ac camundongo protegido	DALTON *et al.*, 1987
53kDa (PDI)	mRNA fêmea	Enzima do tubo digestivo	FINKEN *et al.*, 1994
220kDa - Miosina (SmIrV-5)	Ac coelho α AP	AP protege camundongo Ac paciente, rato e camundongo infectado	NEWPORT *et al.*, 1988a SOISSON *et al.*, 1992
66kDa (SmIrV-1)	Ac coelho α AP	Reconhecido por soro de camundongo protegido	HAWN *et al.*, 1993
70kDa (hsp)	Ac de paciente	Ac rato α *S. japonicum* (= hsp bactéria)	HEDSTROM *et al.*, 1997 e 1988 SCALLON *et al.*, 1987
Sm23	Ac de pacientes	Ag do esquistossômulo	REYNOLDS *et al.*, 1992
Sm14	Ac de camundongos	Protege camundongos contra *S. mansoni* e *Fasciola*	MOSER *et al.*, 1991 TENDLER *et al.*, 1996
37kDa (G3PDH)	Ac coelho α AP	Ac paciente resistente	DESSEIN *et al.*, 1988 GOUDOT-CROZEL *et al.*, 1989
gp200 (GPI ancorada)	Ac coelho α AP	AcM 305 (sinergiza pzq)	BRINDLEY *et al.*, 1989 HALL eta l., 1995
Sm25	Ac coelho α AP	Ac camundongo WEHI (naturalmente resistência) Ac camundongo αTeg	WRIGHT *et al.*, 1988 KNIGHT *et al.*, 1989 OMER-ALI *et al.*, 1991
20-40kDa (SD)	Nucleotídeo	Ac de paciente	SIMURDA eta l., 1988
26kDa (HGPRTase)	Nucleotídeo (= gene camundongo)	Metabolismo das purinas	CRAIG *et al.*, 1988
50k	Ac de coelho α Teg	Ag da membrana do verme jovem	HAVERCROFT *et al.*, 1988
28kDa (ESPG)	Nucleotídeo	mRNA abundante nas fêmeas	KOSTER *et al.*, 1988
107kDa (HMGCoA redutase)	Nucleotídeo	Metabolismo do colesterol	RAJKOVICK *et al.*, 1988
40kDa (tropomiosina)	Nucleotídeo	Ac de paciente e camundongo vacinado	XU *et al.*, 1989
86kDa (hsp)	Ac paciente	Ag de supeficie	JOHNSON *et al.*, 1989
60-65kDa	Ac coelho α AP	Homologia com Ag humano	KHALIFE *et al.*, 1993
Sm26 (GST)	Ac coelho α AP	Purificado com GST	TIU *et al.*, 1988; TROTTEIN *et al.*, 1990; WRIGHT *et al.*, 1991
20kDa e 28kDa	Ac de rato resistente	TP	EL-SHERBEINI *et al.*, 1990
22,6kDa	Ac coelho α AP	Ac camundongo α Teg	JEFFS *et al.*, 1991
gp22	Nucleotídeo		EL-SHEBEINI *et al.*, 1991

Legendas: TP – transferência passiva de imunidade (soro ou Ac); **AcM** – anticorpo monoclonal; **AP** – Ag purificado; **Teg** – Ag de tegumento; **Siglas** (ordem de aparição): **TPI** – triose fosfoisomerase; **CaBP** – protína ligadora de cálcio; **Sm** – Ag do S. *mansoni*; **hsp** – "heat shock protein"; **G3PDH** – gliceraldeído 3-fosfo desidrogenase; **HGPRTase** – hipoxantina guanidinoribosil transferase; **ESPG** – gene codificado da proteína constitutiva da casca do ovo; **HMGCoA redutase** – hidroximetil glutaril coenzima-A redutase; **PDI** – proteína dissulfeto isomerase; **gp** – glicoproteína; **pzq** – praziquantel; **GPI** – glicosil-fosfatidil-inositol; **SD** – superóxido dismutase.

Peptídeos sintéticos da glutationa S-transferase do *Schistosoma mansoni*, um antígeno relevante de 28 kDa, usados em experimentos de imunização, induziram redução da esplenomegalia, do número de ovos depositados nos vasos hepáticos e da fibrose hepática em camundongos infectados pelo *Schistosoma mansoni*. Os mecanismos envolvidos foram evidenciados e não envolveram formação de anticorpos específicos indicando o envolvimento de mecanismos celulares na redução da patologia hepática (PANCRÉ et al., 1994a). Transferência passiva de células esplênicas enriquecidas de células T específicas para estes peptídeos reproduziram o efeito protetor conferido pela imunização e o mecanismo envolvido inclui baixos níveis de IL4 e elevados níveis de IFN-γ (PANCRE et al., 1994a e 1994b).

Tem sido proposto o uso combinado de diferentes Ag como uma estratégia mais promissora, devido à grande complexidade de epitopos envolvidos com as várias formas do parasito. A indução de proteção pode gerar um aumento na resposta granulomatosa responsável pela patologia da esquistossomose. Em geral, a atividade moduladora sobre a reação granulomatosa não tem sido avaliada nos ensaios de proteção. KING *et al.* (1987) verificaram que o granuloma hepático não foi alterado pela imunização. Outros autores (*TAB.* 2, 3a e 3b) avaliam a resposta granulomatosa a partir de ensaios de "footpad-swelling" (SHER *et al.*, 1986) ou de granuloma pulmonar (LUKACS; BOROS, 1991a).

1.5. Justificativa

O papel de componentes do verme adulto do *S. mansoni* na indução de imunidade protetora tem sido amplamente estudado na literatura (SIMPSON; CIOLI, 1985; SHER *et al.*, 1986; GREGOIRE *et al.*, 1987; TENDLER, 1987). Algumas estratégias de imunização experimental usando Ag do verme adulto, como GST (BALLOUL *et al.*, 1987; BOULANGER *et al.*, 1991) e a paramiosina (SHER *et al.*, 1986; PEARCE *et al.*, 1988) ou mesmo a TPI clonada a partir de cercárias (HARN *et al.*, 1987), têm fornecido graus significativos de proteção indicando estes componentes antigênicos como candidatos à vacinação em humanos (WHO, 1991). Entretanto, estes ensaios não tem levado em conta o comprometimento das imunizações na formação do granuloma *in vivo*.

Inúmeros epitopos são compartilhados por diferentes estágios do parasito (DUNNE *et al.*, 1988; GOES *et al.*, 1989; LUKACS; BOROS, 1991b). Além disso, grande quantidade de Ag do verme adulto está constantemente sendo liberada na corrente sanguínea, sendo evidenciada alta reatividade humoral e celular contra estes componentes (DOUGHTY; PHILLIPS, 1982a; TENDLER, 1987; CORREA-OLIVEIRA *et al.*, 1989; BAHIA-OLIVEIRA *et al.*, 1992). Esta reatividade pode estar diretamente envolvida na resposta aos ovos do parasito. Poucos trabalhos de infecção experimental em camundongos (KING *et al.*, 1987; PANCRE *et al.*, 1994a e b) ou macacos (DAMIAN *et al.*, 1984; BOULANGER *et al.*, 1991) avaliaram o papel de componentes do verme adulto na modulação da reação granulomatosa aos ovos do parasito. Além da indução de proteção, normalmente avaliada ao nível da recuperação de vermes adultos em imunizações experimentais e subsequente desafio, seria bastante esclarecedor avaliar o comprometimento da reação granulomatosa com a imunização. O procedimento indutor de imunidade deve evitar ou suprimir a formação de granuloma ao mesmo tempo em que deve induzir imunidade protetora contra as formas larvais e os vermes adultos, protegendo e garantindo o desenvolvimento de um quadro menos severo.

Nossos resultados mostram que Ag de ovo do parasito, reconhecidos por AcM humanos (AcMH) provenientes de pacientes infectados (HIRSCH *et al.*, 1997c) e uma fração aniônica, PIII, obtida a partir do fracionamento da preparação antigênica solúvel de vermes adultos (SWAP) do S. *mansoni* (HIRSCH; GOES, 1996), são capazes de induzir alta proliferação celular e baixa resposta granulomatosa *in vitro* de células do sangue periférico de pacientes esquistossomóticos assintomáticos, forma crônico-intestinal. Acreditamos que componentes da fração PIII possam participar da indução da resposta imunológica característica dos pacientes com esta forma crônica benigna da esquistossomose. Além disso, a imunização experimental com os Ag purificados de extrato antigênico de ovo em cromatografia de afinidade usando estes AcMH (HIRSCH *et al.*, 1997c) e Ag obtidos de extratos antigênicos de vermes adultos, fração PIII (HIRSCH *et al.*, 1997a) foi capaz de induzir proteção contra a infecção experimental em camundongos. Ao mesmo tempo, no caso da fração PIII, ainda foi observada a formação de granulomas hepáticos menores (HIRSCH *et al.*, 1996) e com menor deposição de colágeno (resultados complementares). O efeito de Ag do ovo na indução de proteção, ou seja, na reatividade contra as formas jovens do verme, mostra a intrincada rede de interações entre os Ag dos diferentes estágios evolutivos do parasito e os mecanismos imunológicos do hospedeiro.

A identificação dos componentes de PIII (HIRSCH *et al.*, 1997b) capazes de induzir proteção e regulação da reação granulomatosa, muito poderá contribuir para o entendimento dos mecanismos moduladores do granuloma e no avanço dos estudos rumo a uma vacina eficiente contra esta endemia.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a participação de componentes antigênicos do *Schistosoma mansoni* na indução de imunidade protetora e no estabelecimento da modulação da reatividade granulomatosa dirigida contra os ovos do parasito.

2.2 OBJETIVOS ESPECÍFICOS

- Purificar, por cromatografia de afinidade, e caracterizar os antígenos do extrato solúvel de ovo (SEA) do *Schistosoma mansoni* reconhecidos por anticorpos monoclonais humanos (AcMH) de pacientes esquistossomóticos crônico-intestinais (PCI);
- Avaliar a atividade protetora, *in vivo* e *in vitro*, dos AcMH de PCI;
- Avaliar a indução de imunidade protetora em animais experimentais imunizados com antígenos purificados de SEA usando AcMH;
- Evidenciar a reatividade imunológica *in vitro* de PCI induzida pelos antígenos de SEA purificados com AcMH;
- Fracionar o extrato antigênico de verme adulto (SWAP) do S. *mansoni* em cromatografia de troca aniônica;
- Evidenciar a reatividade imunológica *in vitro* de PCI frente às frações de SWAP obtidas em cromatografia de troca aniônica;
- Avaliar a indução de imunidade protetora e da modulação do granuloma em animais experimentais imunizados com a fração PIII de SWAP;
- Identificação dos componentes de PIII através de tecnologia do DNA recombinante.

3. MATERIAIS E MÉTODOS e RESULTADOS

3.1 Artigos publicados

HIRSCH, C.; GOES, A.M. Characterization of fractionated *Schistosoma mansoni* soluble adult worm antigens that elicit human cell proliferation and granuloma formation *in vitro.* **Parasitol.,** v.112, n.6, p.529-535. 1996.

HIRSCH, C.; ZOUAIN, C.S.; ALVES, J. B.; GOES, A.M. Induction of protective immunity and modulation of granulomatous hypersensitivity in mice using PIII, an anionic fraction of *Schistosoma mansoni* adult worm. **Parasitol.,** v.115, n.1., p.21-28. 1997a.

HIRSCH, C.; CARVALHO, C.Q.; FRANCO, G.R.; PENA, S.D.J.; SIMPSON, A.J.G.; GOES, A.M. Evidence to paramyosin (Sm97) as a modulating antigen on granulomatous hypersensitivity. **Mem. Inst. Oswaldo Cruz,** v.92, n.5, p.663-667. 1997b.

HIRSCH, C.; ALMEIDA, C.A.; DOUGHTY, B.L.; GOES, A.M. Characterization of *Schistosoma mansoni* 44.7/56.8 kDa egg antigens recognized by human monoclonal antibodies which schistosomiasis patients cell proliferation and protection against experimental infection. **Vaccine,** v.15, n.9, p.948-954. 1997c.

Characterization of fractionated *Schistosoma mansoni* soluble adult worm antigens that elicit human cell proliferation and granuloma formation *in vitro*

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SUMMARY

Soluble adult worm antigens (SWAP) of *Schistosoma mansoni* were fractionated by fast protein liquid chromatography (FPLC) system, using Q-Sepharose anion-exchange resin, in order to characterize antigenic fractions that may elicit cell responses in human schistosomiasis. SWAP fractions were eluted by 20 mM Tris–HCl solution (pH 9·6) with an increasing gradient of 1 M NaCl. The FPLC system was able to resolve 6 fractions, enumerated I to VI, according to the NaCl gradient. The analysis of each fraction on SDS–PAGE showed that fractions I to IV were constituted by multiple protein bands with M_r ranging from 21 to > 200 kDa. Large amounts of nucleic acids were evidenced in fractions V and VI, as revealed by ethidium bromide staining of agarose electrophoresis gels. Using ELISA, it was shown that sera from chronic schistosomiasis patients contained antibodies that recognized antigens in practically all fractions. Studies were designed to investigate the capacity of these fractions to induce cell proliferation and granuloma formation. It was demonstrated that fraction III stimulated a significant proliferative response of peripheral blood mononuclear cells (PBMC) from chronic schistosomiasis patients. However, fraction III coupled to polyacrylamide beads induced small granuloma formation *in vitro*, whereas beads coated with fractions I, II and V were able to induce significant granuloma reactions.

Key words: *Schistosoma mansoni*, granuloma, proliferation, human cells.

INTRODUCTION

Schistosomiasis mansoni is a chronic debilitating disease caused by a parasitic helminth, *Schistosoma mansoni* (Phillips & Colley, 1978). The pathology related to infection has been driven by a cell-mediated immune response developed around parasite eggs entrapped mainly in host liver (Boros, Pelley & Warren, 1975; Warren, 1982). Several studies over the last decade have concentrated efforts on the elaboration of a reliable vaccine able to protect humans against infection by *S. mansoni*. This, however, requires thorough identification and characterization of target antigens that provide protective immunity against this parasite. Numerous studies have been conducted in experimental models, in order to demonstrate molecules from the parasite, allowing a better understanding of its immunogenic and biological functions (James *et al.* 1986; Aitken, Coulson & Wilson, 1988; Kambara & Wilson, 1990; Vignali *et al.* 1990). In order to analyse further the molecular basis of host immunity to *S. mansoni*, it is essential to have available defined, purified parasite antigens. Some have been purified from parasite egg, using either conventional purification techniques (Carter & Colley, 1979; Payares *et al.* 1985; Harn *et al.* 1989; Lukacs & Boros, 1992) or affinity chromatography with monoclonal antibodies (Harn *et al.* 1985; Smith & Clegg, 1985; Hsu *et al.* 1986). It has been previously demonstrated that fractions of *S. mansoni* soluble egg antigen were able to sensitize and elicit granulomatous hypersensitivity in mice (Weiss, Aronstein & Strand, 1987; Harn *et al.* 1989; Lukacs & Boros, 1991). However, there is no information available concerning the *S. mansoni* adult worm antigen fractions that mediate granuloma reactivity in humans. In this study, we report the fractionation and characterization of *S. mansoni* adult worm antigens by anionic chromatography on FPLC, to identify those antigens that elicit *in vitro* cell proliferation and granuloma formation in humans.

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MATERIALS AND METHODS

Study population

Patients were selected for active *S. mansoni* infection based on clinical and parasitological stool examination (Hoffman, Pons & Janer, 1934) in Sofia Feldman Hospital, Belo Horizonte, MG, Brazil. The patient protocols used throughout this study were approved by the human subject ethics committee in Brazil.

Antisera

Sera used in this work as antiserum were obtained from normal humans who had never had *S. mansoni* infection diagnosed, from chronically infected humans, and from rabbits immunized by i.m. injections of 100 μg/ml of SEA or SWAP in Complete Freund's Adjuvant, boosted twice at 2-week intervals with identical amounts of antigen, but in Incomplete Freund's Adjuvant.

Antigens

Phytohaemagglutinin (PHA-P; Sigma, St Louis, MO, USA) was used at a concentration of 12·5 μg/ml in culture medium. Antigenic preparations were obtained from schistosome eggs (SEA), adult worms (SWAP) and cercariae (CAP). These antigens were prepared as soluble supernatant fluids from buffered saline homogenates of respective life-cycle stages (Goes *et al.* 1989). Other antigens used were SWAP fractions, obtained by anion-exchange chromatography on FPLC, and bovine serum albumin (BSA). These materials were used based on their protein content to give maximum responsiveness in the blastogenesis assay with most patients (25 μg/ml). All stimulants were sterilized by filtration and stored at −70 °C.

Fractionation of SWAP by Fast Protein Liquid Chromatography (FPLC)

SWAP (30 mg) was dialysed against 20 mM Tris–HCl, pH 9·6, and filtered using an acrodisc 0·2 μm filter. Separation was performed by FPLC (Pharmacia, Uppsala, Sweden) on Q-Sepharose anion-exchange chromatography (5 mm × 90 mm glass columns, packed with Q-Sepharose; bead size distribution: 45–165 μm; Pharmacia). Proteins were eluted with 20 mM Tris–HCl, pH 9·6, in a multistep increasing gradient up to 1 M NaCl, interrupted by hold-gradient intervals at 0, 100, 280, 450, 600 and 750 mM. Flow-through fractions were concentrated by lyophilization. The concentrated material was dialysed against 0·15 M phosphate-buffered saline (PBS), pH 7·4, sterilized by filtration and stored at −70 °C. The protein content of SWAP fractions was measured according to Bradford microassay (Bradford, 1976).

Conjugation of S. mansoni *antigens and BSA to polyacrylamide beads*

Polyacrylamide beads (PB), 40–60 μm in diameter, were obtained from BioRad (Bio-Gel P-4, No. 150-0459; Richmond, CA, USA). Beads were sterilized and washed in 0·5 M bicarbonate buffer by gentle centrifugation. Then 200 mg of beads were incubated for 4 h in a 63 °C water bath with gentle agitation, washed 3 times with sterile distilled water, and then mixed with 20 mg of antigen (BSA, SEA, SWAP or SWAP fractions) and 100 mg of EDAC [(1-ethyl 3-(3) dimethylaminopropyl) carbodiimide HCl] in 100 ml of slightly acidic (pH 6·5–6·8) sterile distilled water. The mixture was gently rotated for 18 h at 4 °C. The beads were then washed in 0·15 M PBS, pH 7·4, and stored at 4 °C in sterile PBS with 0·1% sodium azide until needed.

Cell preparations

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of patients infected with *S. mansoni* or normal individuals. Cells were separated by Ficoll-diatrizoate (LSM, Organon Teknika, Charleston, SC, USA) density-gradient centrifugation (Goes *et al.* 1991), washed and resuspended in culture medium (RPMI 1640 with 10% normal human AB+ serum, 1·6% L-glutamine, 300 U/ml of penicillin, 0·6 mg/ml of streptomycin and 0·05 mg/ml of gentamycin).

In vitro S. mansoni *antigens–polyacrylamide bead cultures*

Two hundred antigen-conjugated polyacrylamide beads were distributed, in a volume of 50 μl, in 96-well tissue culture plates (Linbro, Flow Laboratories, Inc., McLean, VA, USA). Samples of 3×10^5 PBMC from either chronic intestinal schistosomiasis patients, former patients or uninfected controls were added into the culture well, in a final volume of 200 μl of culture medium. Every experimental and control group was set up in triplicate and maintained at 37 °C in a 5% CO_2 incubator. *In vitro* granuloma reactivity was evaluated on day 5 after culture initiation, by quantitation of cellular reactivity around the coated beads (PB-BSA, PB-SEA, PB-SWAP, or PB-SWAP fractions) or uncoated beads (PB), using a phase-contrast inverted tissue culture microscope (Nikon, TMS, Tokyo, Japan). Cellular reactivity was determined by morphological observations based on the following criteria: number of cells binding to the beads, visual evidence of blast-transforming cells accompanied by cellular migration, and adherent cell layers surrounding the beads. A numerical score equivalent to the following classification was assigned to each cell–bead reaction observed: (1) no cells binding to the bead; (2) < 5 cells binding; (3) > 5 cells binding to the bead; (4) > 5 cells binding to the bead accompanied by a circumoval mononuclear cell migration and blast transformation; (5) adherent cell layer attached to the bead accompanied by circumoval mononuclear cell migration; (6) multiple cell layers surrounding the bead accompanied by mononuclear cell migration.

A total of 300 separate determinations was made for each experimental group. The total score was then summed and the resultant mean expressed as Granuloma Index (GI). All antigen bead culture reactivity was compared to the non-specific binding of activated PBMC against polyacrylamide beads not conjugated to antigens (PB) or PB-BSA.

Cell proliferation assays

PBMC proliferation assays in response to PHA, SWAP fractions and other *S. mansoni* antigens (SEA, SWAP and CAP) were done. Briefly, $3{\cdot}0 \times 10^5$ PBMC were cultured in triplicate, in 96-well tissue culture plates in culture medium. Cultures were stimulated with 25 μg/ml of each antigen and maintained at 37 °C in a 5% CO_2 incubator for 5 days. All wells were labelled with 0·5 μCi/well of tritiated thymidine (specific activity, 37 Ci/mM; New England Nuclear, Boston, MA, USA), and cells were collected for scintillation counting 18 h later. Data were calculated as the mean of c.p.m. values of triplicate cultures.

Enzyme-linked immunosorbent assays (ELISA)

To evaluate the specificity of chronic schistosomiasis human sera against SWAP fractions and *S. mansoni* antigens, flat-bottomed microtitre plates (Immulon II, Dynatec Corp., Alexandria, VA, USA) were coated overnight with 100 μl of a 10 μg/ml solution of each antigen in 0·5 M carbonate buffer, pH 9·6. Plates were washed 3 times in 0·05 M PBS containing 0·05% Tween 20 (PBS-T), and blocked with 200 μl of 2% bovine serum albumin (BSA) in PBS, at room temperature. After 1 h of incubation, plates were filled with 100 μl of serial dilutions of infected human sera and re-incubated for 1 h. Plates were washed with PBS-T and incubated for an additional hour with 100 μl of a 1/5000 dilution of goat anti-human Ig peroxidase-conjugated antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). Plates were then washed and peroxidase activity was assayed with 150 μl of *o*-phenylenediamine dihydrochloride (OPD) solution (34 mg of OPD and 20 μl of hydrogen peroxide to 100 ml of citrate/phosphate buffer, pH 5·0). Colour development was stopped with 50 μl of 5% H_2SO_4. The optical density at 492 nm was measured with an automated ELISA reader (Bio-Rad 2550 Reader EIA).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Solubilized SWAP fractions and *S. mansoni* antigen preparations were subjected to discontinuous electrophoresis using SDS–10% polyacrylamide gels, under reducing conditions (Laemmli, 1970). The separated proteins were electrophoretically transferred to nitrocellulose paper (Towbin, Staehelin & Gordon, 1979) and then reacted with rabbit sera against SWAP and SEA.

Statistical analysis

A variance analysis was made using the WILCOXON program of SYSTAT System (Wilkinson, 1989). $P < 0{\cdot}05$ values were considered as significant difference in the experiments.

RESULTS

Fractionation of SWAP by FPLC

SWAP was fractionated on FPLC using a multistep 1 M NaCl gradient. The mobile phase was initiated at 0 mM NaCl (fraction I), increasing the concentration to 100 mM (fraction II), 280 mM (fraction III), 450 mM (fraction IV), 600 mM (fraction V), and finally 750 mM NaCl (fraction VI) (Fig. 1A). Analysis of these fractions separated by 10% SDS–PAGE showed multiple protein bands, most of which fell in M_r ranging from 21 to > 200 kDa (Fig. 1B). Fraction I was shown to be composed of 21 to > 200 kDa proteins. Fraction II was basically constituted of polypeptides in the 21, 28, 36, 52 and 97 kDa range, while fraction III contained high (97 and 160 kDa), intermediate (52 and 56 kDa) and low (28 and 36 kDa) proteins. Components with 28, 36, 52 and 97 kDa were located in fraction IV. Large amounts of nucleic acids were evidenced in fractions V and VI, as revealed by ethidium bromide staining of agarose electrophoresis gels (data not shown).

Antibody reactivity to SWAP fractions

The identification of antigens recognized by anti-SWAP rabbit serum in SWAP fractions was conducted using Western blot analysis. Fig. 2 shows that this serum was reactive against components of 87 and 97 kDa in all fractions. The components of 58 and 76 kDa were recognized in fractions I, III, IV and V. Some components of 36, 52 and 56 kDa were reactive in fraction III. On the other hand, different sera from chronic schistosomiasis patients were tested for their ability to bind to SWAP fraction components. The antibodies present in these sera recognized antigens in practically all fractions as determined by ELISA assay (data not shown). The antibody reactivities were significantly high to fractions I and II. However, a significant decline in antibody recognition of fractions III, IV and V was detected, whereas normal controls for the respective antisera failed to bind to these components.

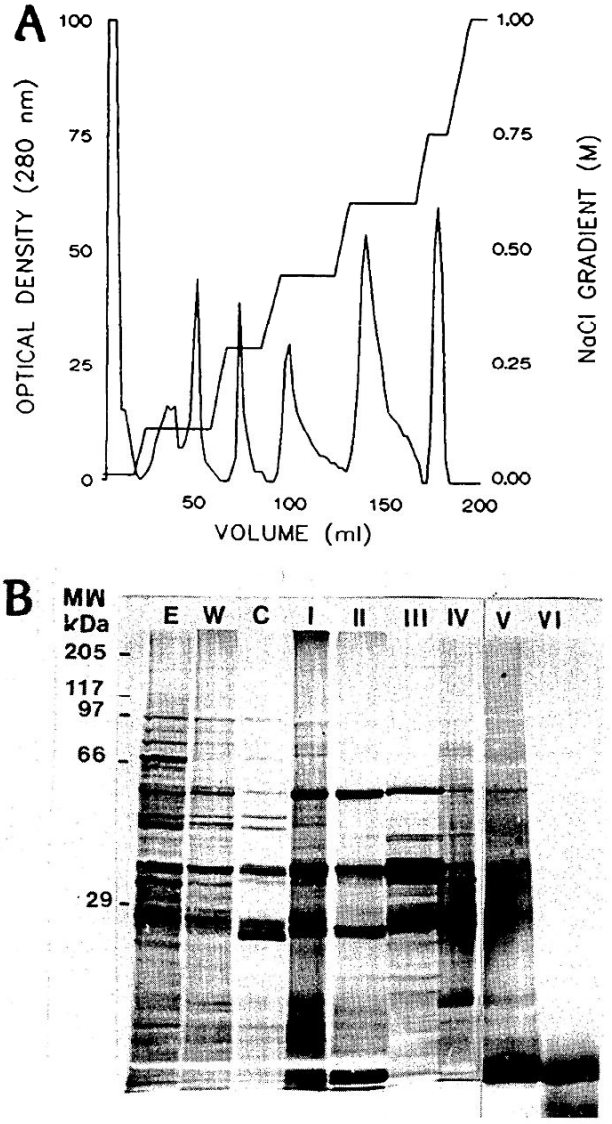

Fig. 1. (A) Elution profile of SWAP on Q-Sepharose anion-exchange column connected to an FPLC system. Proteins were eluted with 20 mM Tris–HCl, pH 9·6, in a multistep increasing gradient, up to 1 M NaCl, interrupted by hold-gradient intervals at 0 mM (Fraction I), 100 mM (Fraction II), 280 mM (Fraction III), 450 mM (Fraction IV), 600 mM (Fraction V) and 750 mM (Fraction VI). (B) SDS–PAGE analysis of SWAP fractions from FPLC and other *Schistosoma mansoni* antigen preparations. Aliquots of 20 μg of each antigenic preparation were separated on 10% polyacrylamide gels, under reducing conditions, followed by silver stain. *S. mansoni* antigens: E-SEA; W-SWAP; C-CAP and SWAP Fractions – I, II, III, IV, V and VI.

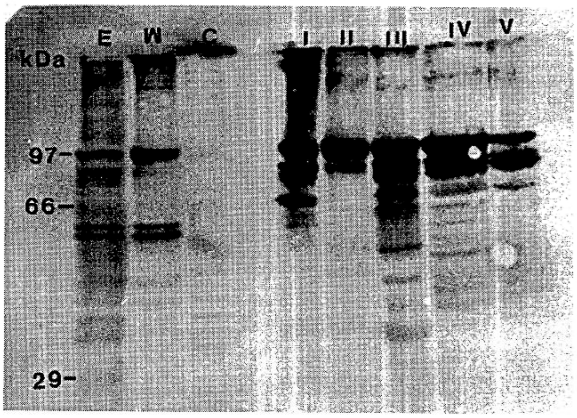

Fig. 2. Western blot analysis of *Schistosoma mansoni* antigens and SWAP fractions, recognized by anti-*S. mansoni* adult worm antigen rabbit serum. Samples prepared from SEA (E), SWAP (W), CAP (C) or SWAP fractions (I–V) were subjected to 10% SDS–PAGE, under reducing conditions, and electrophoretically transferred to nitrocellulose paper. The membrane was incubated with anti-SWAP rabbit serum and revealed with goat anti-rabbit IgG conjugated with peroxidase.

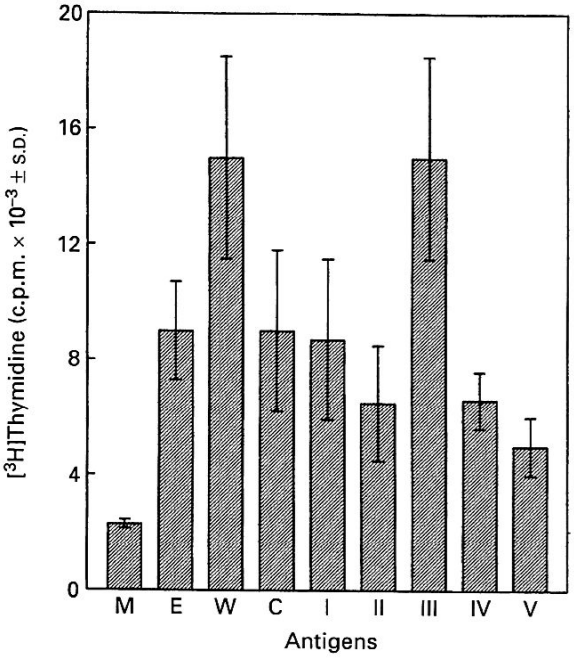

Fig. 3. Proliferative response of PBMC from chronic schistosomiasis patients to *Schistosoma mansoni* antigens. A total of 3×10^5 PBMC were incubated for 5 days, only with medium (M) and with SEA (E), SWAP (W), CAP (C) and SWAP fractions (I–V). Data are reported as means ± S.D. of [^{3}H]thymidine incorporation, in each experimental group ($n = 8$ determinations).

Cellular responses to SWAP fractions

Proliferative responses of PBMC from chronic schistosomiasis patients induced by SWAP fractions are shown in Fig. 3. The results demonstrate considerable variation in degree of proliferative responses between fractions. Components present in fractions I and III elicited significant ($P < 0{\cdot}05$) cell proliferation. However, fraction III gave the highest percentage stimulation. In the remaining fractions examined, we did not detect any significant proliferation induced by fractions II, IV and V. Crude SEA and SWAP promoted strong responses of PBMC, but the proliferation induced by SWAP was the same level as that observed with fraction III.

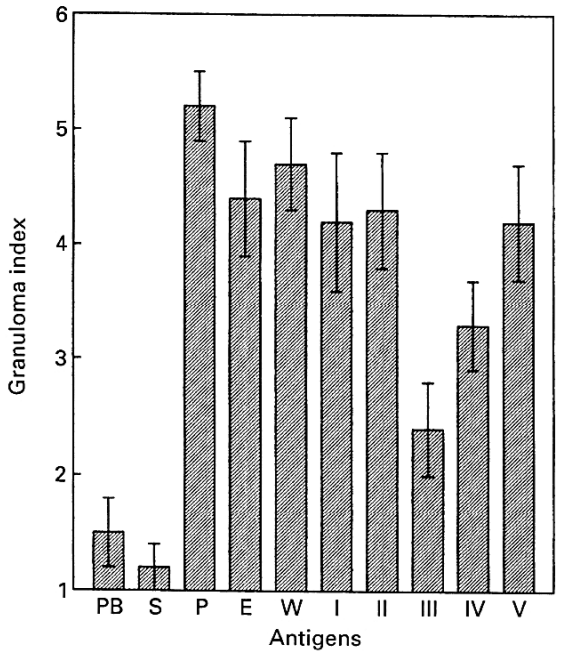

Fig. 4. Granulomatous hypersensitivity response of PBMC from chronic schistosomiasis patients to *Schistosoma mansoni* antigens. A total of 3×10^5 PBMC were incubated for 5 days with polyacrylamide beads (PB), beads conjugated with BSA (S), PPD (P), SEA (E), SWAP (W), and SWAP fractions (I–V). Data are reported as means ± S.D. of granuloma index determined by quantitation of cellular reactivity around the beads ($n = 6$ determinations).

Identification of SWAP fractions that elicit granulomatous responses

Granuloma reaction *in vitro* was performed to determine whether *S. mansoni* adult worm antigens after fractionation were able to elicit granulomatous hypersensitivity. SWAP fractions containing identical amounts of protein were covalently coupled to polyacrylamide beads. Coated beads were used to induce *in vitro* granuloma formation after 5 days of co-cultivation with PBMC from chronic schistosomiasis patients. Significant ($P < 0{\cdot}05$) granuloma formations were observed with fractions I, II and V-coupled beads (Fig. 4), showing several cells around the coated beads. Fractions III and IV elicited small granulomatous responses. However, fraction III induced the lowest granuloma formation ($P < 0{\cdot}001$). Beads coated with crude SEA or SWAP stimulated significantly larger granulomatous reactions ($P < 0{\cdot}05$) than those elicited by fractions III and IV, whereas beads coated with BSA or uncoated beads were not able to induce granuloma reactions.

DISCUSSION

This study showed the results of initial experiments aimed to identify the immunogenic components of *S. mansoni* adult worm antigens essential in schistosomiasis. Our approach to the characterization of granuloma sensitizing-antigens used anionic-exchange chromatography by FPLC to fractionate adult worm antigens (SWAP). In this system, we were able to identify 6 fractions enumerated I to VI depending on the NaCl gradient. These fractions were used to compare both humoral and cellular aspects of chronic schistosomiasis patients' reactivity. In our studies, we identified 2 fractions, I and III, that elicited significant *in vitro* proliferation of PBMC from schistosomiasis patients. The remainder of the fractions were not able to elicit significant and reproducible cell proliferation.

The granuloma reaction of PBMC from these patients to SWAP fraction-coated polyacrylamide beads, and to whole SWAP or SEA-coated beads as positive controls, was also analysed. The data revealed that fractions I, II and V elicited significant granulomatous reactivity of PBMC *in vitro* from a vast majority of the patients tested. Conversely, fractions III and IV were not capable of eliciting significant granulomatous reactivity of patient cells. Results presented here show the possibility that adult worm antigens, besides those from eggs, may be involved in the development of granulomatous hypersensitivity in schistosomiasis, and some of those modulate granulomatous activity. This fact suggests that adult worm antigens play an important role in the development of granuloma reaction to eggs.

Considerable efforts have been exerted to determine which *S. mansoni* antigens induce and elicit T cell-mediated responses and granuloma formation. Several laboratories have isolated various antigens from crude SEA and SWAP, and investigated their roles in serology (Tiu *et al.* 1988; Wright *et al.* 1991), blastogenic reactions (Bahia-Oliveira *et al.* 1992; Contigli *et al.* 1994) and granuloma responses to SEA (Lukacs & Boros, 1991, 1992). Those studies revealed a variety of biologically active antigenic moieties derived from *S. mansoni* antigen preparations. Recently, Lukacs & Boros (1991) fractionated SEA and demonstrated that chronic infection granuloma T cells from mice proliferate to a diminished number of SEA fractions, compared to their acute-infection counterparts. The involvement of cellular responses to antigens derived from adult worms, in the development of typical granulomas in the murine model, was demonstrated *in vitro* by Doughty & Phillips (1982).

The most significant finding reached in the present study is that SWAP components in fraction III were recognized by sera from several patients. In addition, this fraction elicited a proliferative response of PBMC from 25 schistosomiasis patients tested and developed small granuloma formation with granuloma index (GI) around $2{\cdot}3 \pm 0{\cdot}5$ when compared with GI $4{\cdot}8 \pm 0{\cdot}3$ developed against SEA. The relationship of the proteins presented on fraction III to

the modulation of granuloma reactivity in chronic schistosomiasis patients is not clear at present. However, immunization experiments in mice with fraction III showed 50% decrease of granuloma size in liver after challenge infection (data not shown). It appears that several different molecules present in SWAP fractions are responsible to induce the heterogeneity in cell-mediated responsiveness. This indicates the existence, within adult worm antigens, of several molecules that might play a role in activation and modulation of granulomatous hypersensitivity to eggs. In the light of the heterogeneity found among the various SEA fractions that induced cell-mediated responsiveness, Carter & Colley (1979) and Boros, Tomford & Warren (1977) proposed that several biochemically different molecules are involved in granulomatous hypersensitivity. Several studies have demonstrated that granuloma formation induced by egg antigens in murine schistosomiasis included fractions with 70 kDa (Hamburger *et al.* 1982; Lustigman, Mahmoud & Hamburger, 1985); > 200, 54 and 37 kDa (Harn *et al.* 1989), and > 200, 93–125, 70–90, 60–66, 50–56 and 40–46 kDa (Lukacs & Boros, 1991); and in human schistosomiasis, 84, 63, 55–57, 40, 30 and 28 kDa (Gaafar *et al.* 1993).

Conversely, the experiments performed by Gaafar *et al.* (1993) were not capable of eliciting granulomatous reactivity of PBMC from patients with urinary schistosomiasis stimulated with adult worm antigens. However, in our studies, SWAP fractions separated by FPLC proved to be useful reagents, as their soluble or immobilized forms allowed quantitation and interfraction comparisons of lymphocyte proliferation, granuloma formation and regulation (manuscript in preparation), respectively. In conclusion, this study represents our initial efforts aimed to identify *S. mansoni* adult worm components responsible for host sensitization and granulomatous hypersensitivity in human schistosomiasis mansoni.

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Induction of protective immunity and modulation of granulomatous hypersensitivity in mice using PIII, an anionic fraction of *Schistosoma mansoni* adult worm

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SUMMARY

This study was performed in order to define *Schistosoma mansoni* antigens that are able to function as modulator agents in the granulomatous hypersensitivity to parasite eggs in BALB/c and C57BL/6 mice. A fraction of *S. mansoni*, designated PIII, derived from adult worm antigen preparation (SWAP) was obtained using anion-exchange chromatography on an FPLC system. Immunization of mice with PIII in the presence of *Corynebacterium parvum* and $Al(OH)_3$ as adjuvant induced an immune response in these animals as determined by ELISA and spleen cell proliferation assays against *S. mansoni* antigens SEA, SWAP and PIII. In addition, PIII caused a significant degree of protection against a challenge infection in immunized mice as observed by the decrease on worm burden recovered from the portal system. We also showed that PIII profoundly inhibited the vigorous anamnestic granulomatous response to eggs in the liver and lungs. This suppression correlated with a significant decrease in granuloma size. From these results we conclude that the PIII preparation contains antigens that can mediate protective anti-parasite immunity and downregulate granulomatous hypersensitivity to *S. mansoni* eggs.

Key words: *Schistosoma mansoni*, modulation, granuloma, worm, immunity.

INTRODUCTION

Parasite egg-granulomas are the primary pathogenic lesions in experimental and human schistosomiasis mansoni (Warren, 1982; Boros, 1989). This cell-mediated granulomatous response is specific for soluble egg antigen and appears to be mediated predominantly by CD4+ Th2 cells (Grzych *et al.* 1991; Chensue *et al.* 1992). As infection progresses from the acute to the chronic phase, the cell-mediated anti-soluble egg antigen response attenuates in a process called modulation. Experimental murine schistosomiasis has been widely used to investigate parasite biology, host–parasite interactions, including immunology and morbidity, and strategies for treating or preventing this common tropical disease of man (Phillips & Colley, 1978). Therefore, the identification of *Schistosoma mansoni* antigens and an assessment of their role in host–parasite interaction, notably in the prevention or decrease in size of granuloma formation by immunization procedure is essential for the development of an anti-schistosome vaccine (Bergquist, 1990). Future progress in this field might depend on the understanding of the complex immunoregulatory events that modulate the evolution of granulomatous hypersensitivity to *S. mansoni* eggs and the basis of protective immunity in man (Butterworth, 1992). Various experimental approaches have been carried out by investigators to identify *S. mansoni* antigens that elicit T cell proliferation and granuloma formation. These include parasite antigens purified by conventional purification techniques (Carter & Colley, 1979; Payares *et al.* 1985; Harn *et al.* 1989; Lukacs & Boros, 1992), affinity chromatography with monoclonal antibodies (Harn *et al.* 1985; Smith & Clegg, 1985; Hsu *et al.* 1986; Dissous, Grzych & Capron, 1982; Goes *et al.* 1989) or by recombinant DNA technology (Boulanger *et al.* 1991; Jeffs *et al.* 1991). Several aspects of the model used have not been adequately defined because of conflicting results reported by different laboratories, notably the type of antigens which yield the highest levels of resistance, a single or cocktail antigen that gives significantly increased resistance and whether such a vaccine would aggravate or ameliorate the development of granulomatous egg pathology (Warren, Domingo & Cowan, 1967). Our laboratory has produced PIII, an anionic fraction from soluble adult worm antigen preparation (SWAP). This fraction was able to induce high levels of proliferation and small granuloma formation *in vitro* by human peripheral blood mononuclear cells from chronic schistosomiasis patients (Hirsch & Goes, 1996). We have extended these studies in this paper to

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investigate whether PIII would protect mice and modulate granuloma formation resulting from challenge schistosome infections.

MATERIALS AND METHODS

Mice and parasites

Adult female BALB/c and C57BL/6 mice purchased from Centro de Bioterismo, ICB, UFMG, Brazil, were used throughout this study. The mice were maintained under standard laboratory care. The cercariae of *S. mansoni* were obtained from *Biomphalaria glabrata* snails previously infected with miracidia of the L.E. strain, from Belo Horizonte, Brazil. Cercariae were shed from infected snails under bright artificial illumination. Anaesthetized mice (8/group) were exposed to normal cercariae on the abdomen by using the ring method of Smithers & Terry (1965). Vaccinated and control mice challenged with normal cercariae were perfused 8 weeks after exposure (Smithers & Terry, 1965). The protective activity of each group assayed was evaluated by comparing the difference between the recoveries of worms from immunized and control mice.

Antigens

Concanavalin A (Con-A, Sigma, St Louis, MO, USA) was used at a concentration of 12·5 μg/ml in culture medium. Antigenic preparations were obtained from schistosome eggs (SEA) and adult worms (SWAP). These antigens were prepared as soluble supernatant fluids from buffered saline homogenates of the respective life-cycles stages (Goes *et al.* 1989). Other antigens used were PIII, obtained from SWAP by anion-exchange chromatography on an FPLC system, and bovine serum albumin (BSA). These materials were used based on their protein content to give the maximum response in blastogenesis assays with most mice (25 μg/ml). All stimulants were sterilized by filtration and stored at −70 °C.

PIII preparation

PIII was prepared as previously described by Hirsch & Goes (1996). Briefly, SWAP (30 mg) was dialysed against 20 mM Tris–HCl, pH 9·6, and filtered using an acrodisc 0·2 μm filter. Separation was performed by FPLC (Pharmacia, Uppsala, Sweden) on Q-Sepharose anion-exchange chromatography (5 mm × 90 mm glass columns, packed with Q-Sepharose; bead size distribution 45–165 μm; Pharmacia). Proteins were eluted with 20 mM Tris–HCl, pH 9·6, in a multistep increasing gradient up to 1 M NaCl, interrupted by hold-gradient intervals at 0 (PI), 100 (PII), 280 (PIII), 450 (PIV), 600 (PV) and 750 mM (PVI). Flow-through fractions were concentrated by lyophilization. The concentrated material was dialysed against 0·15 M phosphate-buffered saline (PBS), pH 7·4, sterilized by filtration and stored at −70 °C. The protein content of SWAP fractions was measured according to the Bradford microassay (Bradford, 1976).

Immunization of mice with PIII

BALB/c and C57BL/6 were immunized by s.c. injections of 10 μg PIII in the presence of 100 μg of *Corynebacterium parvum* and 1 mg of aluminium hydroxide [$Al(OH)_3$] as adjuvant. The animals were boosted twice at 2-week intervals with identical amounts of antigen, but the last injection was done by i.p. injection without adjuvant.

Induction and measurement of pulmonary granulomas elicited by S. mansoni *eggs*

The induction of synchronous egg granulomas was performed as described previously (Lukacs *et al.* 1994). Briefly, *S. mansoni* eggs were extracted from the livers of infected mice. To induce granulomas, normal, infected or PIII-immunized mice were injected i.v. with 2000 intact living *S. mansoni* eggs. After 4 days, animals were killed and their lungs were inflated and fixed with 4% paraformaldehyde in phosphate buffer and then embedded in paraffin. Sections were stained with haematoxylin and eosin or Heidenhein's azan (Hirata *et al.* 1993). Only lesions with a single well-defined egg nidus were measured. The results were expressed as mean of area (μm^2) calculated from 30–50 granulomas.

Measurement of hepatic granuloma formulation

PIII-immunized and infected mice were killed 8 weeks after challenge infection and the livers were removed and fixed with 4% paraformaldehyde in phosphate buffer. Histological sections were stained with haematoxylin and eosin or Heidenhein's azan (Hirata *et al.* 1993). The areas of granulomas surrounding single, mature eggs were measured by the same procedure used for pulmonary granuloma determination.

Spleen cell preparation

Single-cell suspensions, which had been prepared from spleens of normal, infected or PIII-immunized mice, were treated with Tris–ammonium chloride, pH 7·2, to lyse erythrocytes, and then were washed and counted. The spleen cells were resuspended in culture medium (RPMI 1640 with 20% foetal bovine serum, 1·6% L-glutamine, 300 U/ml of penicillin, 0·6 mg/ml of streptomycin and 0·05 mg/ml of gentamycin).

Table 1. Antibody response and cellular reactivity induced in mice immunized with PIII

Sera and spleen cells from C57BL/6 mice	Antibody reactivity*			Cellular reactivity†		
	SWAP‡	PIII	SEA	SWAP	PIII	SEA
Normal	0·083	0·203	0·210	0·800	0·635	0·927
Chronic infection	1·012	1·698	2·000	4·327	3·734	8·004
PIII-immunized	1·230	0·977	0·699	13·513	7·133	12·836

* Antibody reactivity determined by ELISA assay. Data are reported as means of optical density at 1/640 serum dilution in each experimental group performed twice ($n = 5$ determinations).
† Cellular reactivity was determined by cell proliferation assay. Data are reported as means $\times 10^3$ cpm of [^{3}H]thymidine incorporation in each experimental group performed twice ($n = 8$ determinations).
‡ SWAP, Adult worm antigen preparation; PIII, anionic fraction derived from SWAP; SEA, soluble egg antigen.

Table 2. Protective effect of PIII on vaccinated mice against a challenge infection with *Schistosoma mansoni* cercariae

(Protective effect of PIII was studied in BALB/c and C57BL/6 mice immunized with 10 μg of antigen in the presence of *Corynebacterium parvum*/Al(OH)$_3$ as adjuvant.)

Treatment	Worm burden*	
	C57BL/6	BALB/c
Infection	45±2	40±4
Adjuvant	42±4·4	38±6
PIII	25±6†	25±4†
% Reduction‡	44	37

* Worm burden was determined by perfusion of portal system at 8 weeks after challenge infection in 8 mice/group performed 3 times.
† $P < 0{\cdot}05$.
‡ %Reduction = 100 × control − experimental/control.

Cell proliferation assays

Spleen cell proliferation assays in response to Con-A, PIII and other *S. mansoni* antigens (SWAP and SEA) were performed. Briefly, $7{\cdot}0 \times 10^5$ spleen cells were cultured in 200 μl of culture medium in 96-well flat-bottomed plates. Every experiment was set up in triplicate. Cultures were stimulated with 25 μg/ml of each antigen and maintained at 37 °C in a 5% CO_2 incubator for 3 days. For the last 18 h of incubation, 0·5 μCi/well of tritiated thymidine (specific activity, 37 Ci/mmol; New England Nuclear, Boston, MA, USA) were pulsed. The cells were harvested for scintillation counting and the data were calculated as the mean of cpm values.

Enzyme-linked immunosorbent assays (ELISA)

To evaluate the specificity of (i) normal, and (ii) *S. mansoni* chronically infected and immunized mice sera against PIII, flat-bottomed microtitre plates (Immulon II, Dynatec Corp., Alexandria, VA, USA) were coated overnight with 100 μl of a 10 μg/ml solution of each antigen (SWAP, PIII and SEA) in 0·5 M carbonate buffer, pH 9·6. Plates were washed 3 times in 0·05 M PBS containing 0·05% Tween 20 (PBS–T), and blocked with 200 μl of 2% bovine serum albumin (BSA) in PBS, at room temperature. After incubation for 1 h, plates were filled with 100 μl of serial dilutions of mice sera and re-incubated for 1 h. Plates were washed with PBS–T and incubated for 1 additional hour with 100 μl of a 1/5000 dilution of goat anti-mouse Ig peroxidase-conjugated antibody (Sigma, St Louis, MO, USA). Plates were then washed and peroxidase activity was assayed with 150 μl of *o*-phenylenediamine dihydrochloride (OPD) solution (34 mg of OPD and 20 μl of hydrogen peroxide to 100 ml of citrate/phosphate buffer, pH 5·0). Colour development was stopped with 50 μl of 5% H_2SO_4. The optical density at 492 nm was measured with an automated ELISA reader (Bio-Rad 2550 Reader EIA).

Statistical analysis

Data were analysed statistically by the Student's *t*-test with the level of significance set at $P < 0{\cdot}05$.

RESULTS

Antibody response induced by PIII

Mice were immunized with PIII using *C. parvum* and Al(OH)$_3$ as adjuvant, and the immune response was analysed by ELISA assay. The data from Table 1 show that PIII immunization induces a high antibody response against PIII and SWAP. The antibody reactivity for PIII and SWAP determined in sera from PIII-immunized mice was in the same level as observed for sera from chronically infected ones. We observed that sera from infected mice

Table 3. Reduction of granulomatous hypersensitivity of mice immunized with PIII

	Granuloma area*			
	Hepatic		Pulmonary	
Conditions	C57BL/6	BALB/c	C57BL/6	BALB/c
Non-infected	—	—	7·1 ± 4·1	3·7 ± 2·5
Infected	77·6 ± 39·1	108·5 ± 55·1	39·1 ± 14·1	32·1 ± 20·5
PIII-immunized	42·6 ± 24·3†	61·7 ± 30·0†	19·1 ± 8·5†	9·9 ± 8·6†
%Reduction‡	45	43	51	69

* Granuloma area was calculated assuming a spherical shape. The results were reported as the mean $\times 10^3$ $\mu m^2 \pm$ S.D. from 30–50 granulomas for each mouse of 2 separated experiments ($n = 5$ mice/group).
† $P < 0{\cdot}05$.
‡ %Reduction = 100 × control − experimental/control.

present a significant reactivity to SEA. However, this was not observed in sera from PIII-immunized mice. Normal mice sera did not react with either SEA, SWAP or PIII. On the other hand, sera from mice vaccinated with PIII without adjuvant or with adjuvant only did not present any reaction with all tested antigens (data not shown). Therefore, in the following experiment we concentrated on the further analysis of cellular activity induced by PIII.

Stimulatory capacity of PIII for S. mansoni*-specific spleen cells* in vitro

Splenic cells from infected and PIII-immunized mice were tested for proliferative responses to *S. mansoni* antigens SEA, SWAP and PIII. The results in Table 1 demonstrate considerable variation in degree of proliferative responses between the antigens tested. Crude SEA promoted strong responses of splenic cells from chronically infected mice and PIII-immunized mice. We observed that SWAP induced a significant ($P < 0{\cdot}05$) proliferation of cells from infected mice but this was not at the same level as observed to SEA. A significant cell proliferation was also induced by PIII in the same group of mice. PIII was subsequently tested in proliferation assays with cells from PIII-immunized mice. The data indicated that the proliferation induced by PIII was smaller than that obtained with SWAP or SEA on cells from immunized mice (Table 1). Spleen cells derived from non-infected mice did not respond to any *S. mansoni* antigens used.

Immunization of mice with PIII and its protective effect on a subsequent infection with S. mansoni *cercariae*

The protective effect of immunization with PIII in the presence of *C. parvum* and $Al(OH)_3$ as adjuvant was additionally investigated in mice. As shown in Table 2 the number of worms recovered 8 weeks after challenge infection of PIII-immunized mice was significantly reduced when compared to that observed in adjuvant only or with non-immunized control animals. Immunization with PIII established a protection of 44 and 37 % respectively to C57BL/6 and BALB/c.

Immunization of mice with PIII and its effect on pulmonary and hepatic granuloma formation

Immunization of mice with PIII resulted in a significant reduction in pulmonary granuloma size of BALB/c and C57BL/6 subsequently injected with *S. mansoni* eggs (Table 3). The granuloma reaction was tightly compact and focal aggregates of cells were observed around or close to intravascular and intra-alveolar eggs. We did not detect any focal aggregation in normal mice injected with eggs only (Fig. 1). The sizes of pulmonary granulomas of vaccinated mice were $9{\cdot}9 \times 10^3$ and $19{\cdot}1 \times 10^3$ μm^2 to BALB/c and C57BL/6 respectively, and those infected with cercariae alone were $32{\cdot}1 \times 10^3$ and $39{\cdot}1 \times 10^3$ μm^2 (Table 3). The percentages of granuloma reduction in these mice were 69 and 51 %. Comparison of means showed very significant differences between any two experimental treatments. In addition, we investigated the effect of PIII immunization in hepatic granuloma reaction after challenge infection. The foci in the livers of infected mice with cercariae were more numerous and frequently very large (Fig. 2), extending to up to 77×10^3 μm^2 and 108×10^3 μm^2 respectively in C57BL/6 and BALB/c (Table 3). The hepatic granulomas in the PIII-vaccinated group presented small foci and the size was reduced to $42{\cdot}6 \times 10^3$ μm^2 and $61{\cdot}7 \times 10^3$ μm^2 for C57BL/6 and BALB/c (Table 3). The percentages of granuloma reduction in these animals were 45 and 43 %. Each was significantly different from the controls at $P < 0{\cdot}05$.

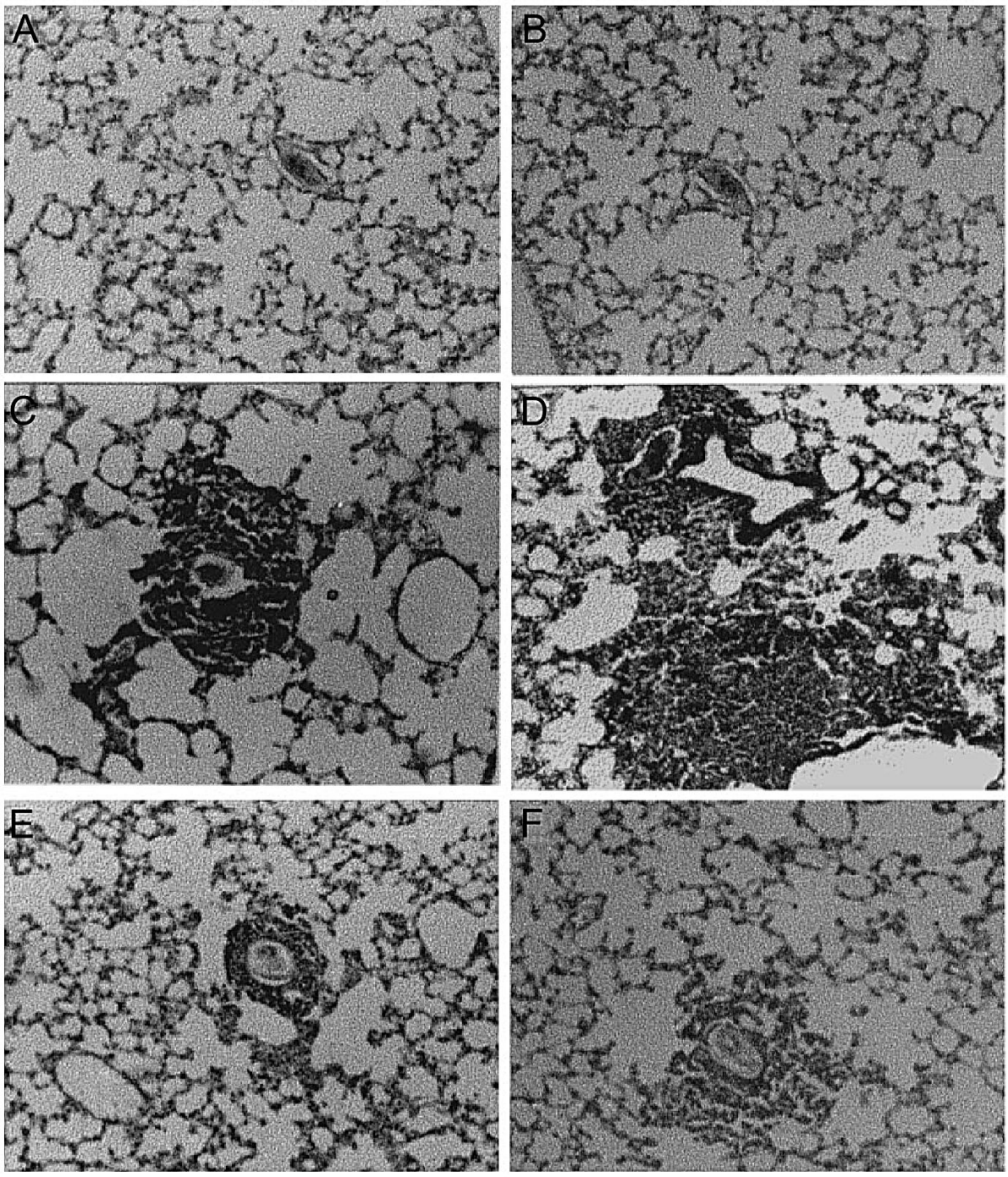

Fig. 1. Pulmonary granuloma photomicrographs (×250) of BALB/c (A, C, E) or C57BL/6 (B, D, F) mice after 4 days of i.v. egg injection in either normal (A, B), chronically infected (C, D) or immunized with PIII (E, F) mice. Histological sections were stained with haemotoxylin–eosin.

DISCUSSION

The present study describes an antigenic protein fraction PIII, obtained from *S. mansoni* adult worms, which fails to induce vigorous granulomatous hypersensitivity to eggs on liver and lungs, but also causes protection against a challenge infection in mice after local (s.c.) immunization. Our results demonstrated that mice vaccinated with PIII can alter the formation of granulomatous hypersensitivity to the parasite eggs. The vaccinated mice developed pulmonary and hepatic granulomas that were statistically smaller than those formed in the unvaccinated controls. In addition, spleen cells from these mice had a strong proliferative response to SEA and SWAP and even to PIII. It appears that PIII components are responsible for inducing heterogeneity in cell-mediated responses. These

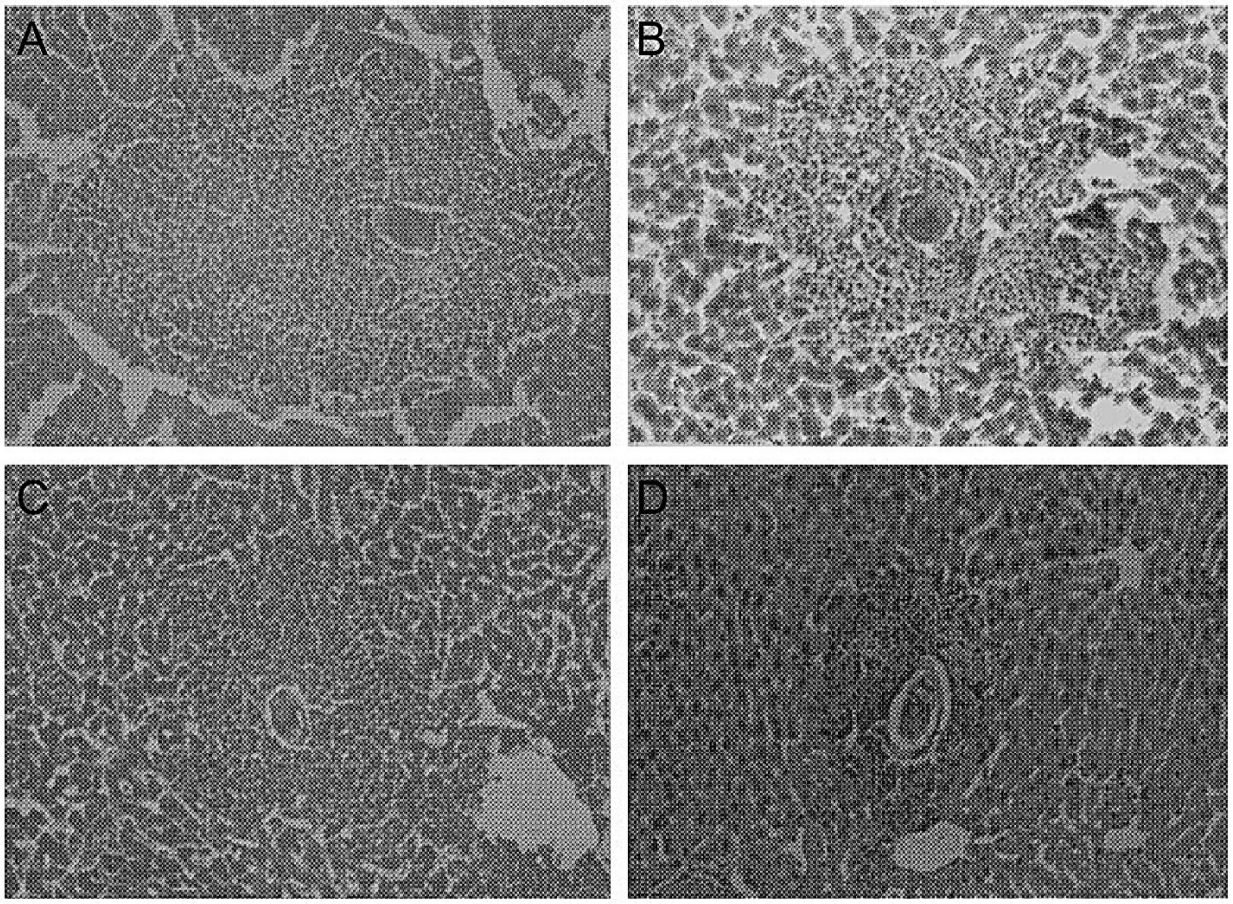

Fig. 2. Hepatic granuloma photomicrographs (×250) of BALB/c (A, C) or C57BL/6 (B, D) mice 8 weeks after challenge infection in either non-immunized (A, B) or PIII immunized mice (C, D). Histological sections were stained with haematoxylin–eosin.

findings are in accord with previous results reported both by Hirsch & Goes (1996), using an *in vitro* granuloma formation system and cell proliferation of peripheral blood mononuclear cells from human schistosomiasis patients, and by Wynn *et al.* (1994) in mice. The ability of PIII to suppress both pulmonary and hepatic egg-induced granulomatous responses suggested the possibility of prophylactically immunizing mice against granulomatous inflammation by sensitizing them with adult worm antigens in the presence of *C. parvum* and $Al(OH)_3$. This indicated the presence, within adult worm antigens, of several molecules that might play a role in cell activation and modulation of granulomatous hypersensitivity. The existence of an SEA cross-reactive humoral immune response prior to egg deposition is well documented (Dissous & Capron, 1983; Goes *et al.* 1989). In contrast, there is a scarcity of data on T cell cross-reactivity between larval, adult worms and egg antigens. Early publications reported a lack of granulomatous responsiveness in mice injected intravenously with eggs or immunized with irradiated cercariae (Warren, 1982). Recently, Contigli *et al.* (1994) demonstrated that human T cell clones selected from PBMC stimulated with SEA preferentially proliferated in response to SWAP or CAP and that these clones were able to induce *in vitro* granulomas in cooperation with autologous antigen-presenting cells.

Conversely, post-schistosomal hepatic lesions have been shown to be caused by the formation of egg granulomas in the portal area. However, studies done by others suggest the contribution of adult worm antigen in part to the development of the hepatic lesion. The essential role of adult worms in the formation of egg granulomas *in vitro* (Doughty & Phillips, 1982; Hirsch *et al.* 1997) and the strong association between vigorous response to adult worm antigen and the development of hepatic lesions (Deelder *et al.* 1980; Elner *et al.* 1980; Ohta *et al.* 1982) support this concept, although it had been assumed that the strength of the immune response to the adult worm antigen depends on the intensity of infection. However, additional studies by Barsoum *et al.* (1982) demonstrated that the responsiveness to the adult worm antigen is determined mainly by immunoregulatory mechanisms. Indeed, there is considerable debate concerning the immunological effector mechanisms responsible for the rejection of challenge parasites in resistant animals with evidence

supporting a function for humoral responses (Capron & Capron, 1994) and other findings indicating a role for cell-mediated immunity (James & Boros, 1994). Therefore, an important strategy for improving vaccine efficacy is to define mechanisms of immunity operating in a given vaccination protocol and to attempt to enhance the response for young parasite forms and decrease the granulomatous hypersensitivity to eggs trapped in host tissue.

The data presented here clearly demonstrate that PIII might be of help in the formulation of a vaccine which both reduces worm burden and inhibits the pathology resulting from egg deposition. Examination of the production of circulating anti-PIII antibody revealed an increase in levels in PIII-immunized mice. Since antibody production was maintained in the face of moderate inflammation, this tends to argue in favour of the notion of antibody playing a direct role in the regulation of granuloma formation (Goes *et al.* 1991; Parra *et al.* 1991; Rezende *et al.* 1993).

A recent report of vaccines against schistosomiasis (Butterworth, 1992) showed that certain facts or aspects of the host immune response were important for the practical evaluation of the usefulness of schistosomiasis vaccines. When vaccines have been tried in mice, the end result has always been that a few organisms of a challenge infection develop into adult worms. Because adult worms do not divide, any antigen candidate that will reduce the number of worms and the granuloma size in humans would be beneficial (Colley & Colley, 1989; Butterworth, 1992). It is also possible that a vaccine candidate in humans will be a cocktail of antigens that will be used to protect against schistosomiasis. Therefore, PIII has this advantage because this fraction is constituted by 5 principal components (Hirsch & Goes, 1996). The immunization with a mixture of PIII in *C. parvum*/$Al(OH)_3$ and administered subcutaneously is capable of inducing significant parasite killing mechanisms and downregulation of granulomatous hypersensitivity involving stimulation of both humoral and cell-mediated immunity against further infection without exacerbating cell-mediated tissue pathology.

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Evidentiation of Paramyosin (Sm-97) as a Modulating Antigen on Granulomatous Hypersensitivity to *Schistosoma mansoni* Eggs

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A Schistosoma mansoni *adult worm anionic fraction (PIII) has previously been shown to protect mice against challenge infection and to reduce pulmonary and hepatic granulomatous hypersensitivity. Serum from PIII-immunized rabbit was used to screen a λgt11 cDNA library from* S. mansoni *adult worm in order to identify antigens capable of modulating granulomatous hypersensitivity. We obtained four clones with 400 (Sm-III.11), 900 (Sm-III.16), 1100 (Sm-III.10) and 1300 (Sm-III.12) bp of length. All clone-specific antibodies were able to recognize most of the PIII components. The sequence analysis showed that these clones presented high homology with* S. mansoni *paramyosin (Sm-97). These findings ascribe a new function to this antigen with an important role in modulation of granulomatous hypersensitivity to* S. mansoni *eggs.*

Key words: Paramyosin - modulation - granuloma - *Schistosoma mansoni*

Schistosomiasis mansoni pathology is ascribed to an inflammatory response towards parasite eggs trapped in host tissues (von Lichtenberg 1987, Boros 1989). The intensity of this response can be modulated by host (Ottensen 1979, Goes et al. 1991) and parasite factors (Ellner et al. 1981, Boulanger et al. 1991). Worms and eggs share the same environment and many adult worm antigens are constitutively shedded in flow blood (Simpson et al. 1984). These antigens can crossreact with egg components (Dunne et al. 1988, Goes et al. 1989) and could be able to modulate the immune response against eggs.

On experimental models, the protective immunity has been studied using a number of antigens isolated from parasite forms (Simpson & Cioli 1987, Butterworth 1992). Many of these parasite components were able to induce moderate levels of protection (Harn et al. 1987, Sher et al. 1986). However, these studies did not analyze the induced granulomatous reactivity to eggs. Besides protection, if granuloma formation could be prevented or suppressed, the development of severe disease might be averted (Boros 1989).

Our laboratory has produced an adult worm anionic fraction (PIII) by separation of adult worm antigen preparation (SWAP) using anionic exchange chromatography on FPLC system (Hirsch & Goes 1996). Immunization of mice with PIII in the presence of *Corynebacterium parvum* and $Al(OH)_3$ as adjuvant induced an immune response that caused a significant protection degree against a challenge infection (Hirsch et al. 1997). In addition, we also showed that PIII induced a significant decrease in granuloma formation to eggs in the liver and lungs of vaccinated mice (Hirsch et al. 1997). Therefore, the interest in the understanding of immunobiology of granuloma response and the role of purified PIII components in the induction of regulatory events that modulate the granulomatous hypersensitivity to *S. mansoni* eggs, has directed our efforts to the characterization of PIII components at molecular level. In this study, we report the identification of *S. mansoni* cDNA clones which encode adult worms antigens recognized by rabbit serum specific for PIII.

MATERIALS AND METHODS

Antisera - Serum was obtained from rabbits immunized by i.m. injections of 100 μg/ml of PIII or SWAP in Complete Freund Adjuvant, boosted

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twice at two weeks intervals with identical antigen amounts, but in Incomplete Freund Adjuvant.

Antibody select solutions were prepared by plating out phage clone of interest, inducing fusion protein expression by overlaying the plates with isopropyl-β-D-thiogalactopyranoside (IPTG)-soaked nitrocellulose filters, then incubating these filters containing adherent fusion protein overnight in anti-SWAP or anti-PIII rabbit sera. Affinity selected antibodies were then eluted from the filters with 0.1 M glycine buffer pH 2.6, the eluates pooled, neutralized with Tris-HCl, pH 8.0 and fetal calf serum added to 10%.

PIII preparation - PIII was prepared as previously described by Hirsch and Goes (1996). Briefly, SWAP (30 mg) was dialyzed against 20 mM Tris-HCl, pH 9.6, and filtered using an acrodisc 0.2 μm filter. Separation was performed by FPLC (Pharmacia, Upsala, Sweden) on Q-Sepharose anion-exchange chromatography (5 mm x 90 mm glass columns, packed with Q-Sepharose; bead size distribution: 45-165 μm; Pharmacia). Proteins were eluted with 20 mM Tris-HCl, pH 9.6, in a multistep increasing gradient up to 1 M NaCl, interrupted by hold-gradient intervals at 0 (PI), 100 (PII), 280 (PIII), 450 (PIV), 600 (PV) and 750 mM (PVI). Flow-through fractions were concentrated by lyophilization. The concentrated material was dialyzed against 0.15 M phosphate-buffered saline (PBS), pH 7.4, sterilized by filtration and stored at -70°C. The protein content of SWAP fractions was measured according to Bradford microassay (Bradford 1976).

Sodium duodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting - PIII and *S. mansoni* antigen preparations (SWAP) were subjected to discontinuous electrophoresis using SDS-10% polyacrylamide gels, under reducing conditions (Laemmli 1970). The separated proteins were electrophoretically transferred to nitrocellulose paper (Towbin at al. 1979) and then reacted with rabbit sera against SWAP and PIII.

cDNA library screening - An adult worm cDNA library, constructed in the λgt11 vector was screened (Jeffs et al. 1991) using rabbit serum against PIII, the protective anionic fraction from SWAP. Positive phage plaques were selected and re-screened to obtain pure clones. Clone inserts were amplified by Polymerase Chain Reaction (PCR) using λgt11 primers adjacent to the *Eco*RI cloning site (λgt11F: 5'-ggtggcgacgactcctgg agcccg-3' and λgt11R: 5'-ttgacaccagaccaact ggtaatg-3'). The amplification reaction mixture contained 1 μl of the phage suspension in SM buffer, 0.2 μM of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs and 1U Taq DNA polymerase (Promega) in a 20 μl final volume of a specific reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0 and 0.1% Triton X-100). The thermal profile comprised 30 cycles of denaturation at 96°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. Amplification products were analyzed on ethidium bromide stained 1% agarose gels.

DNA sequencing and homology searches- Phage inserts from clones Sm-III.10, Sm-III.11, Sm-III.12, Sm-III.16 were PCR amplified using the biotinylated forward primer (λ31F: 5'-gaaggcacatggctgaatatc-3') and the reverse primer (λ183R: 5'-gcgaaatacgggcagaca-3') in a final volume of 100 μl, following the amplification protocol described above. The amplification products were purified using streptavidin coated beads (Dynal). DNA was alkali denatured and the immobilized strand was used for dideoxy chain-termination sequencing with the nested λgt11R primer, essentially as described by Hutman et al. (1989). The sequencing reactions were performed as suggested in the Auto Read Sequencing kit (Pharmacia LKB). The sequencing products were submitted to electrophoresis on a Pharmacia A.L.F. Automated DNA Sequencer. Partial sequences obtained were searched for homology with DNA and protein sequences deposited in databases using the Basic Local Alignment Search Tool (BLAST) (Altshul et al. 1990) e-mail server at the National Center for Biotechnology Information (NCBI) site.

RESULTS

Anti-PIII rabbit serum was able to recognize, by Western blot analysis, components of 160, 97, 45 and 36 kDa after separation on 10% SDS-PAGE under reducing conditions (Figs 1, 2).

This serum was used to screen a λgt11 cDNA library constructed from *S. mansoni* adult worms. Various positive phage plaques were selected and re-screened in order to obtain pure clones (Fig. 3). Four phage preparations were shown to be pure after two re-screenings and all clones contained only one insert after PCR amplification. The sizes of the four inserts were 400 (Sm-III.11), 900 (Sm-III.16), 1100 (Sm-III.10) and 1300 (Sm-III.12) bp long (Fig. 4).

Antibodies specific to the clones were obtained by overnight incubation of filters containing pure phage plaques expressing fusion proteins with anti-PIII polyclonal rabbit serum. Affinity selected antibodies were eluted and used on Western blot assays. These four antibody select solutions recognized most of PIII components separated on reducing conditions in 10% SDS-PAGE (Fig. 5).

The phage inserts were PCR amplified using the biotinylated forward primer (λ31F) and the reverse primer (λ183R) and submitted to solid-phase

sequencing. Partial sequences obtained after single-runs on the A.L.F. DNA sequencer were searched for homology with all DNA and protein sequences deposited in non-redundant databases. All sequences were shown to presented 100% homology with *S. mansoni* paramyosin.

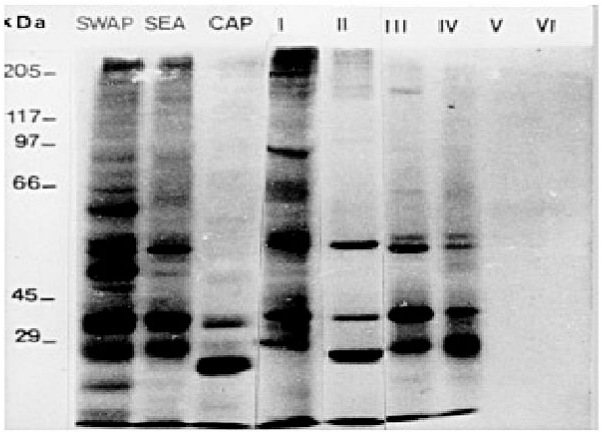

Fig. 1: SDS-PAGE analysis of SWAP fractions from FPLC and other *Schistosoma mansoni* antigen preparations. Aliquots of 20 µg of each antigenic preparation were separated on 10% polyacrylamide gels, under reducing conditions, followed by silver staining. *S. mansoni* antigens: SWAP; SEA; CAP and SWAP fractions - I, II, III, IV, V and VI.

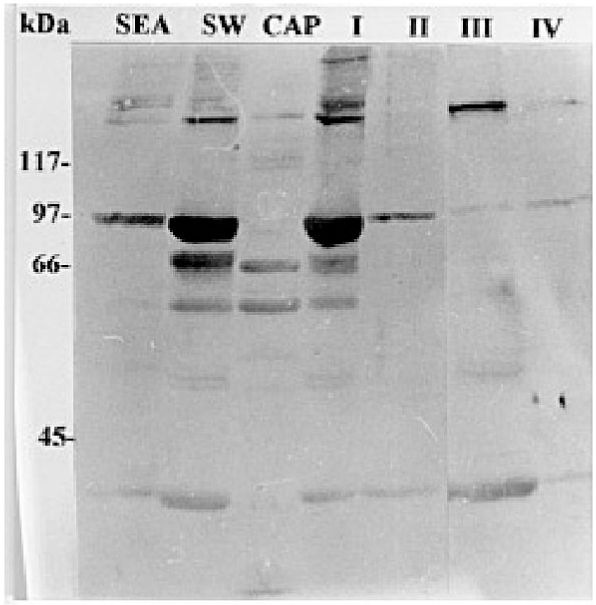

Fig. 2: Western blot analysis of *Schistosoma mansoni* antigens and SWAP fractions, recognized by anti-PIII rabbit serum. Samples prepared from SEA, SWAP (SW), CAP or SWAP fractions (I-IV) were subjected to 10% SDS-PAGE, under reducing conditions, and electrophoretically transferred to nitrocellulose paper. The membrane was incubated with anti-PIII rabbit serum and revealed with goat anti-rabbit IgG conjugated with peroxidase.

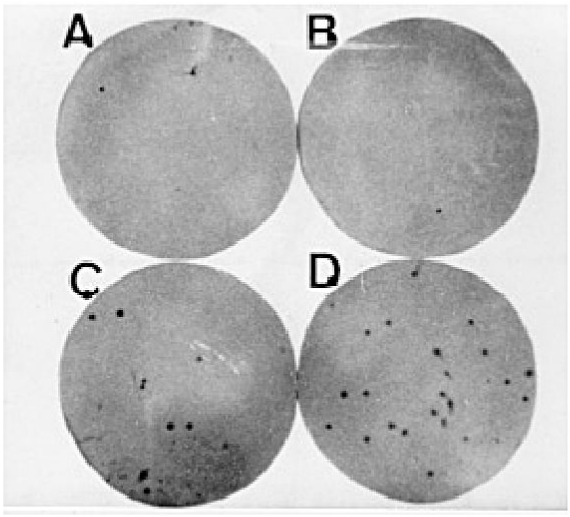

Fig. 3: identification and cloning of *Schistosoma mansoni*-λgt11 recombinants expressing antigenic polypeptides. The plaques were screened with antibodies from rabbit immunized with PIII (A - 1st screen, C-2nd screen, D-clone) or from normal rabbit serum (B). The positive clones were identified using BICP/NBT by incubating with goat anti-rabbit Ig conjugated directly to alkaline phosphatase.

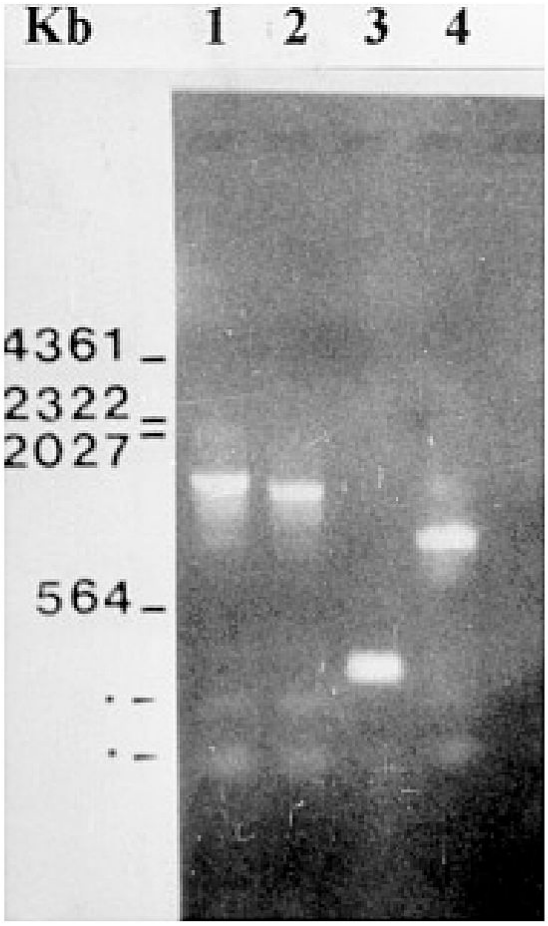

Fig. 4: PCR amplification of *Schistosoma mansoni*-cDNA clones. Ten microlliters of each sample were electrophoresed on 1% agarose containing ethidium bromide (0.5 µg/ml). Lane 1- Sm-III.12 clone; lane 2- Sm-III.10 clone; lane 3- Sm-III.11 clone and lane 4- Sm-III.16 clone.

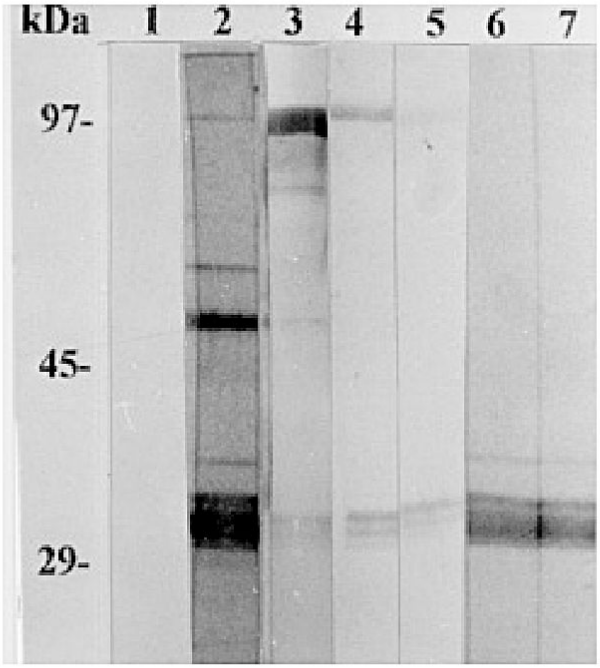

Fig. 5: Western blot analysis of SWAP and PIII probed with antibody selecting solutions against Sm-III.10 and Sm-III.11 clones. SWAP incubated with normal rabbit serum (lane 1); SWAP (lane 2) and PIII (lane 3) incubated with anti-PIII rabbit serum; SWAP (lane 4) and PIII (lane 5) incubated with antibody selected against Sm-III.10 clone; SWAP (lane 6) and PIII (lane 7) incubated with antibody selected against Sm-III.11. Antibodies were probed with a goat alkaline phosphatase-conjugated anti-rabbit Ig.

DISCUSSION

In previous studies on the search of antigens that mediate modulation of granulomatous hypersensitivity against *S. mansoni* eggs, we reported the use of an antigenic protein fraction, obtained from *S. mansoni* adult worm, PIII. This fraction fails to induce vigorous granulomatous hypersensitivity to eggs on liver and lungs, and causes protection against a challenge infection in mice after local (s.c.) immunization. The ability of PIII to suppress both pulmonary and hepatic egg-induced granulomatous responses suggested the possibility of prophylatically immunizing mice against granulomatous inflammation by sensitizing them with adult worm antigens in the presence of *C. parvum* and $Al(OH)_3$. This indicates the presence, within adult worm antigens, of several molecules that might play a role in cell activation and modulation of granulomatous hypersensitivity.

Various experimental approaches have been carried out by investigators to identify target antigens that provide protective immunity and participate as granulomatous modulating agent in schistosomiasis (Harn et al. 1987, Simpson & Cioli 1987, Boulanger et al. 1991, Hirsch & Goes 1996). We believe that molecular cloning and sequencing analysis is an important strategy to identify candidate vaccine antigens against *S. mansoni*.

The data presented here describe four cDNA clones Sm-III.10, Sm-III.11, Sm-III.12 and Sm-III.16 encoding immunologically recognized polypeptides that correspond to proteins contained in PIII, an anionic fraction obtained from SWAP (Hirsch & Goes 1996). The utilization of antibodies specific to the products of these clones was able to identify SWAP proteins with molecular weight of 97 and 35 kDa by western blot analysis. Partial nucleotide sequences of these clones showed 100% of homology with *S. mansoni* paramyosin.

Paramyosin (Sm-97) is the most abundant antigen on *S. mansoni* adult worm. Studies have shown that it is able to induce protection against challenge infection in mice model (Sher et al. 1986). As GST, paramyosin was recognized by reinfection resistant human sera (Correa-Oliveira et al. 1989). Sm-97 was already cloned (Lanar et al. 1986) and its structure is well known (Sher et al. 1986, Lanar et al. 1986, Pearce et al. 1986). Although its capability to induce granulomatous hypersensitivity in footpad swelling assay (Pearce et al. 1986), our studies showed shorter hepatic granuloma reaction induced in immunized mice with PIII (Hirsch & Goes 1996), where paramyosin is a prevalent antigen as we showed in this paper.

In summary, using a powerful rabbit antiserum to screen an adult worm cDNA library, up to 240,000 PFU were analyzed and for four times we obtained clones coding for the muscular protein paramyosin. These findings suggest a prevalence of this antigen in PIII anionic fraction and our results ascribe a role for paramyosin in granuloma modulation.

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Characterization of *Schistosoma mansoni* 44.7/56.8 kDa egg antigens recognized by human monoclonal antibodies which induce protection against experimental infection and proliferation of peripheral blood mononuclear cells from schistosomiasis patients

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We described here the characterization of Schistosoma mansoni *egg antigens recognized by human monoclonal antibodies B10 (HmAb-B10) and D5 (HmAb-D5). SDS–PAGE and Western blot analysis revealed that these monoclonals recognized two antigens of M W 44.7/56.8 kDa, with pI of 7.0 and 7.8, respectively. The passive transfer of B10 and D5 induced a significant protection of 48% and 54% in Balb/c mice. Results of* in vitro *cytotoxicity assay showed that both monoclonals were able to kill schistosomula in the presence of rabbit complement. These monoclonals mediated 48% and 74% of schistosomula cytotoxicity, respectively. Egg antigens were purified by affinity chromatography using monoclonal antibodies B10 and D5. Treatment of purified antigens with periodate, galactose oxidase and trifluoromethane sulphonic acid did not prevent binding by B10 and D5 in ELISA assay. However, the treatment with protease K and 2-mercaptoethanol affects the antibodies binding, showing that the HmAbs B10 and D5 recognize polypeptide epitopes. Vaccination of mice with these antigens in Freund's adjuvant induced 43% reduction in worms burden after challenge with* S. mansoni *cercariae.* In vitro *blastogenesis assays with peripheral blood mononuclear cells from patients infected with* S. mansoni *revealed that purified antigens were able to induce significant cell proliferation.*

Keywords: vaccine; antigen; proliferation; purification of *Schistosoma mansoni* egg antigens

Schistosomiasis mansoni is a parasitic disease that affects millions of people in certain regions of Africa and Latin America[1]. It is caused by a parasitic helminth, the *Schistosoma mansoni*, and is characterized by the presence of adult worms in the portal and mesenteric veins of man and other mammalian species[2]. An important goal of studies on the host immune response against *S. mansoni* is the development of an effective vaccine to prevent infection by this parasite[3,4]. Various experimental approaches have been carried out by investigators to identify target antigens that provide protective immunity in schistosomiasis[5–8]. One vaccine candidate is the 38 kDa glycoprotein present in schistosomula and adult worm metabolic products that had been implicated in protective immunity[9]. An additional 28 kDa protein (GST), identified in metabolically labeled adult worms, on *in vitro* translation products and on schistosomula surface, was able to induce both IgE and IgG protective antibodies and conferred up to 70% of resistance to

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challenge infection[10]. A 97 kDa protein (paramyosin), isolated from soluble *S. mansoni* worm antigen preparation, when injected intradermally with BCG, was able to induce significant levels of protection (35–70%) to challenge infection[11]. Our laboratory produced human monoclonal antibodies selected for further studies on the basis of their reactivity to *S. mansoni* antigens[12]. Two selected monoclonals, HmAb-B10 and HmAb-D5, recognized two major components on *S. mansoni* SEA preparation with 44.7 kDa and 56.8 kDa, as evidenced in Western blot assay[12]. We have purified the egg antigens recognized by HmAb B10 and D5, examined them chemically and then determined their capacity to induce protection against experimental infection and to elicit proliferation of peripheral blood mononuclear cells (PBMC) from schistosomiasis patients.

MATERIAL AND METHODS

Study population

Schistosomiasis patients were selected based on clinical and parasitological stool examinations[13] in Hospital Sofia Feldman, Belo Horizonte, MG, Brazil. The patient protocols used throughout this study were approved by the human subject ethics committee in Brazil.

Antisera

Sera used in this work as antisera were obtained from humans chronically infected with *S. mansoni*, from uninfected humans, and from rabbit immunized by intramuscular (i.m.) injections of 200 μg ml^{-1} affinity purified antigen with human monoclonal antibodies (HmAb) B10 and D5[12] in complete Freund adjuvant, boosted three times at 2 weeks intervals with identical antigen amounts, but in incomplete Freund adjuvant.

Antigens

Phytohemaglutinin (PHA-P; Sigma, St. Louis, MO, USA) was used at a concentration of 12.5 μg ml^{-1} in a culture medium. Antigenic preparations were obtained from schistosome eggs (SEA), adult worms (SWAP) and cercariae (CAP). These antigens were prepared as soluble supernatant fluids from buffered saline homogenates of respective life-cycle stages[12]. Other antigens used were Ag-B10 and Ag-D5, purified from SEA by chromatography using HmAb-B10 and D5, which recognize 44.7 and 56.8 kDa molecules. These materials were used based on their protein content to give maximum responsiveness in a blastogenesis assay with most patients (25 μg ml^{-1}). All stimulants were sterilized by filtration and stored at −70°C.

Cell preparations

PBMC were isolated from heparinized blood of patients infected with *S. mansoni* or normal individuals. The cells were separated by Ficoll-diatrizoate density gradient centrifugation (LSM Organon-Teknika, Charleston, SC, USA)[14], washed and resuspended in culture medium (RPMI 1640 with 10% normal human AB+ serum, 1.6% L-glutamine, 300 U ml^{-1} of penicillin, 0.6 mg ml^{-1} of streptomycin and 0.05 mg ml^{-1} of gentamycin).

Cell proliferative assays

PBMC proliferative assays in response to PHA, affinity purified 44.7/56.8 kDa SEA antigens and other *S. mansoni* antigens (SEA, SWAP and CAP) were done. Briefly, 3×10^5 PBMC were stimulated in culture medium with 25 μg ml^{-1} of each antigen in triplicate and maintained in 96-well tissue culture plates, at 37°C in 5% CO_2, for 5 days. Plate wells were labeled for 18 h with 0.5 μCi well^{-1} of tritiated thymidine (sp. act. of 37 Ci mM^{-1}; New England Nuclear, Boston, MA, USA), and cells were harvested for scintillation counting. Data were calculated as mean of c.p.m. values of replicate cultures.

In vitro cytotoxicity assay for schistosomula

Antibody-dependent complement cytotoxicity assays were carried out on schistosomula mechanically transformed[15]. Briefly, 100 μl of either HmAb-B10 and D5 at 1 mg ml^{-1} concentration, 100 μl of normal pooled human sera or 100 μl of pooled sera collected from schistosomiasis patients were added to 100 newly transformed schistosomula in 50 μl Elac medium[15] in triplicate wells of 96-well flat bottomed plates. Rabbit serum, as a source of complement, was added to all wells in a volume of 100 μl (diluted 1:2 v/v with Elac). Cultures were incubated overnight in a humidified atmosphere with 5% of CO_2 and viable schistosomula were counted after the addition of Trypan Blue. Schistosomula were considered dead if they absorbed the blue stain, showed no movement, and exhibited dense granularity of internal cellular components.

Passive transfer experiments

Groups of 8 Balb/c mice (8-week-old female) were injected i.v. with either 1 mg/0.5 ml of HmAb-B10, HmAb-D5 or normal human IgM. As a positive control was used 0.5 ml of chronic schistosomiais human serum. One hour later, all groups were percutaneously infected with 100 *S. mansoni* cercariae. Parasite burdens were evaluated 8 weeks later by the liver perfusion technique according to Smithers and Terry[16]. The number of liver worms obtained from mice injected with B10 and D5 was compared with those obtained from mice injected with normal human IgM. The percentage of protection was calculated by the formula $[(c-e)/c] \times 100$, in which c is the number of worms recovered from mice injected with normal human IgM and e is the number of worms recovered from mice injected with schistosomiais chronic serum, B10 or D5.

Enzyme-linked immunosorbent assays (ELISA)

Flat bottomed microtiter plates (Immulon II, Dynatec Corp., Alexandria, VA) were coated overnight with 100 μl of a 10 μg ml^{-1} solution of *S. mansoni* antigens and of affinity purified SEA antigens in 0.5 M carbonate buffer, pH 9.6. The plates have been washed three times in 0.15 M PBS containing 0.05% Tween 20 (PBS-T), and were blocked with 200 μl of 1% bovine serum albumin (BSA) in PBS-T at room temperature. After 1 h of incubation, the plates were filled with 100 μl of serial dilutions of rabbit serum against affinity purified SEA antigen or HmAb and re-incubated for 2 h. Plates were washed with PBS-T and incubated for an

additional 1 h with 100 μl of a 1:1000 dilution of goat anti-rabbit Ig peroxidase conjugated antibody or 1:5000 dilution of goat anti-human Ig peroxidase conjugated antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). The plates were washed and the peroxidase activity was assayed with 150 μl of *o*-phenylenediamine dihydrochloride (OPD) solution (34 mg of OPD and 20 μl of hydrogen peroxide to 100 ml of citrate/phosphate buffer pH 5.0). The color development was stopped with 50 μl of 5% H_2SO_4. The optical density at 492 nm was measured with an automated ELISA reader (Bio-Rad 2550 Reader EIA).

Periodate-ELISA

Periodate-ELISA was carried out by modifying the ELISA assay before the incubation with HmAb. Antigen-coated plates were washed three times with PBS-T, and blocked with 2% BSA in PBS-T overnight. Further, the plates were washed three times with PBS-T, and 100 μl of 20 mM sodium periodate in 50 mM acetate buffer were added to each well[17]. After 1 h at 25°C, the reaction was stopped by adding 100 μl of 50 mM sodium borohydride and 100 μl of 1% glycine solution in PBS-T, and incubated for 30 min. Then, the plates were washed three times with PBS and the assay continued as described before.

Galactose oxidase—ELISA

Galactose oxidase (Sigma Chemical Co., ST. Louis, MO, USA) was heated at 56°C for 1 h and stored at −70°C until use. The plates were coated with *S. mansoni* antigens and treated with 50 μl of galactose oxidase at 0.3 U μl^{-1} concentration at 37°C overnight. The reaction was stopped by adding 100 μl of selected samples of bis(2-hydroxyethyl) dithiocarbamate, an inhibitor of galactose oxidase (2/1 v/v in isopropanol)[18]. After 16 h, the enzyme was removed by washing the plates three times in PBS-T. HmAb binding was determined as already described. For trifluoromethanesulfonic acid (TFMS) antigen treatment, we used the method described by Edge *et al.*[19]. Briefly, 1 ml of TFMS/anisole (2/1 v/v) was added to 100 μl of 0.2 mg ml^{-1} of SEA for 30 min on ice. After stopping the reaction with 10% ethanol, the antigen was diluted to 10 μg ml^{-1} with 0.5 M of carbonate buffer pH 9.0, distributed in 96-well plates and incubated overnight. The plates were washed three times in PBS-T, and the monoclonal antibody binding was determined as described before.

Purification of Ag-B10 and Ag-D5 sea by affinity chromatography

Human monoclonal antibodies B10 and D5 were purified from concentrated culture supernatants by affinity chromatography, using Protein A–Sepharose for IgG or ABx columns for IgM and IgG3[20]. Purified antibodies were coupled to cyanogen bromide-activated Sepharose 4B[21]. Ten milligrams of SEA were applied to columns, and chromatography performed with 20 mM Tris–HCl, pH 7.5 in 500 mM NaCl. The elution of the bound proteins was performed with 2 M KSCN, 20 mM Tris–HCl pH 7.5. The eluated were dialyzed against 3 mM phosphate buffer, pH 7.4. Purified antigens were lyophilized, redissolved in 0.15 M PBS, pH 7.4 and sterilized by filtration.

Table 1 Susceptibility of *S. mansoni* to human monoclonal antibodies

Source of antibodies	*In vivo*		*In vitro*
	Worm burden[a]	% Protection	% of schistosomula killing
C-inactivated	N.D.	N.D.	5
NH-IgM	42±8	—	6
CH-serum	29±5	31*	38*
B10	22±4	48*	48*
D5	19±9	54*	74*

*$P<0.05$. [a]$N=8$ mice per group performed twice. C-Rabbit, inactivated serum; NH-IgM, normal human purified IgM; CH-serum, human schistosomiasis, serum from chronic infection; B10-HmAb, B10 IgM isotype reactive to *S. mansoni*; D5-HmAb, D5 IgM isotype reactive to *S. mansoni*; % protection=100×control–experimental/control

Protection assays

Groups of five Balb/c mice were immunized by i.m. injections of 10 μg of SEA antigens affinity purified by HmAb-B10 in complete Freund's adjuvant (v/v), boosted twice at 2 week intervals with identical antigen amounts, but in incomplete Freund's adjuvant. Two weeks after the third injection, the mice were infected percutaneously with 100 cercariae. The worms' recovery and evaluation of the percentage of protection were performed as described before. Mice controls were either injected with BSA in adjuvant or with adjuvant alone.

SDS–PAGE and Western blotting

SDS polyacrylamide gels (10%) were run under non-reducing conditions according to the methods of Laemmli[22]. Separated proteins were transferred electrophoretically onto nitrocellulose paper[23] and then labeled with human monoclonal antibodies[12].

Statistical analyses

Data were analyzed statistically by the Student's *t*-test with the level of significance set at $P<0.05$.

RESULTS

In vitro and *in vivo* killing of *S. mansoni* mediated by human monoclonal antibodies

We have previously described the generation of human monoclonal antibodies which reacted with *S. mansoni* egg antigens[12]. Two of them, B10 and D5, were selected for studies of cytotoxicity to schistosomula *in vitro* and parasite killing *in vivo*. When mechanically transformed schistosomula were incubated overnight in the presence of 20 μg ml^{-1} of human monoclonal antibodies, we found that B10 and D5 were capable of killing 48% and 74% of parasites, respectively (*Table 1*). By comparison, only 5% of the organisms were killed by the inactivated normal rabbit serum, while IgM from uninfected individuals killed <6%. Pooled chronic human schistosomiasis human serum produced 38% of killing. The protective role of B10 and D5 *in vivo* was

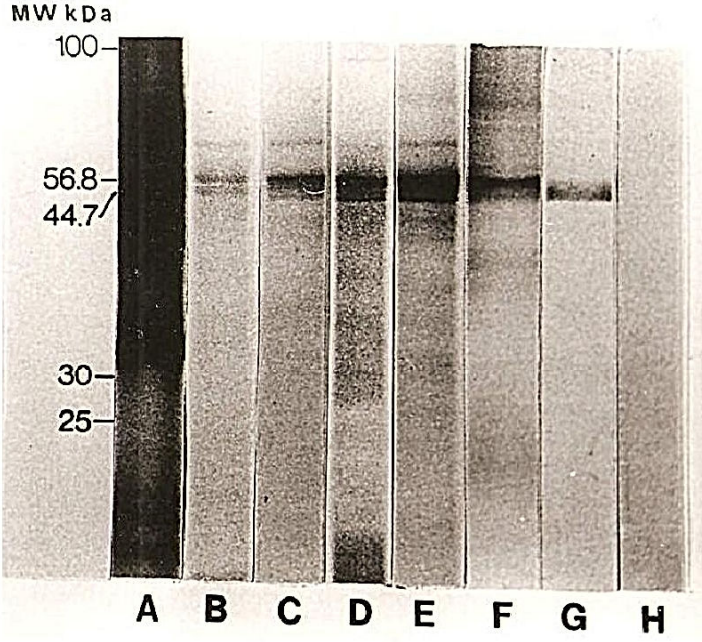

Figure 1 SDS–PAGE and Western blot of *S. mansoni* affinity purified 44.7/56.8 kDa egg antigens. Samples were applied to 10% SDS–PAGE under non-reducing conditions followed by silver stain (lines B and C). The separated proteins were transferred onto nitrocellulose. The strips were labeled with HmAb B10 (line D) and HmAb D5 (line E); with rabbit sera against 44.7/56.8 kDa (lines F and G). Crude egg antigens (SEA) were labeled with human chronic sera (line A) or labeled with normal human IgM (line H)

also investigated by passive transfer experiments. The results, presented in *Table 1*, show a lower number of worms recovered from mice treated with B10 (22+4) and D5 (19+9). From those mice injected with normal human IgM, 42 ± 8 worms were recovered. In our experimental conditions, the level of protection transferred with B10 was 48%, and with D5, 54%.

Immunoaffinity purification of egg antigens of *S. mansoni* recognized by human monoclonal antibodies

Immunoaffinity chromatography was used to purify antigens from SEA prepared in the absence of protease inhibitors. We have used SDS–PAGE and Western blot analysis to show that monoclonal antibodies B10 and D5 columns were able to purify 44.7/56.8 kDa SEA antigens (*Figure 1*). Two-dimensional electrophoresis demonstrated that these purified antigens present pI of 7.0 and 7.8, respectively (data not shown).

Biochemistry characterization of epitopes recognized by B10 and D5

In order to establish the biochemical nature of the epitopes on 44.7/56.8 kDa SEA recognized by HmAb B10 or D5, enzymatic and chemical modification experiments were undertaken. It was demonstrated that the antigen could be partially or completely digested by overnight treatment with proteinase K (73% binding reduction) or 2-mercaptoethanol (2-ME; around 92% binding reduction), respectively (*Table 2*). However, the addition of PMSF, an inhibitor of proteinase K, significantly affected the loss of antibody binding to these antigens. The effect of deglycosylation on the binding of HmAb B10 and D5 to SEA antigens is also shown in *Table 2*. We observed that the binding of both B10 and D5 was not reduced by treatment with 50 mM periodate at acid pH, galactose oxidase or TFMS.

Table 2 Binding effect of human monoclonal antibodies to 44.7/56.8 kDa egg antigens after chemical and enzymatic epitope modifications

	Antibody binding and % of reduction (ELISA assay O.D.)			
Antigen treatment	CH-S	D33	B10	D5
None	1.50	0.10	0.60	0.60
Protease K	1.30 13%	0.08	0.17* 72%	0.16* 73%
PK-PMSF	1.50	0.10	0.80 NR	0.79 NR
2-ME	1.30 13%	0.07	0.06* 90%	0.30* 95%
PMSF	1.70	0.05	0.80 NR	0.82 NR
SP	1.40	0.07	1.30 NR	0.95 NR
GOX	1.50	0.05	0.84 NR	0.89 NR
TMSF	1.40	0.08	0.63 NR	0.71 NR

*$P<0.05$. PK, Protease K; PMSF, protease K inhibitor; 2-ME, 2-mercaptoethanol; SP, sodium periodate; CH-S, human schistosomiasis: serum from chronic infection; B10 and D5, HmAb IgM isotype reactive to *S. mansoni*; GOX, galactose oxidase; D33, SHM-D33 supernatant; TMSF, trifluoromethane sulphonic acid. % Reduction=100×control–experimental/control

Cellular response to affinity purified antigens

PBMC from patients with chronic intestinal schistosomiasis were tested for *in vitro* proliferation with 44.7/56.8 kDa SEA antigens. These cells showed vigorous proliferation to these antigens in the concentrations of 3, 5 and 25 $\mu g\ ml^{-1}$ (*Figure 2*). However, the cells proliferated preferentially in response to the antigen purified by HmAb D5. In the same assay, PBMC were highly stimulated by SEA and SWAP, while CAP elicited a low degree of proliferation. No proliferation was observed when PBMC from normal individuals were stimulated with these antigens.

Effect of 44.7/56.8 kDa egg antigens administration

The protective effect of immunization with 44.7/56 kDa antigens was additionally investigated in mice in the presence of Freund's adjuvant. As shown in *Table 3*, the number of worms recovered after 8 weeks of infection of 44.7/56.8 kDa immunized mice was significantly reduced compared with that observed in BSA-immunized mice or in non-immunized control animals. The immunization with purified egg antigens established a protection of 43%.

DISCUSSION

We used two human IgM monoclonal antibodies, designated HmAb-B10 and HmAb-D5 to characterize, and purify a SEA major antigen of *S. mansoni*. In order to characterize the functional properties of these monoclonal antibodies, we conducted investigations on the passive transfer of protection against challenge infection to *S. mansoni*, and tested the *in vitro* cytotoxicity activity of these HmAbs against schistosomula. The monoclonals gave high level protection in passive transfer experiments. When schistosomula were incubated with B10 and D5 antibodies in the presence of the rabbit complement, we detected a high level of cytotoxicity induced by D5, and moderate level by B10. The protection conferred by the transfer of B10 and D5 was closely correlated with the degree of *in vitro* cytotoxicity by the same antibodies, thus supporting the relationship between *in vitro* cytotoxicity and *in vivo* protection in the mouse model.

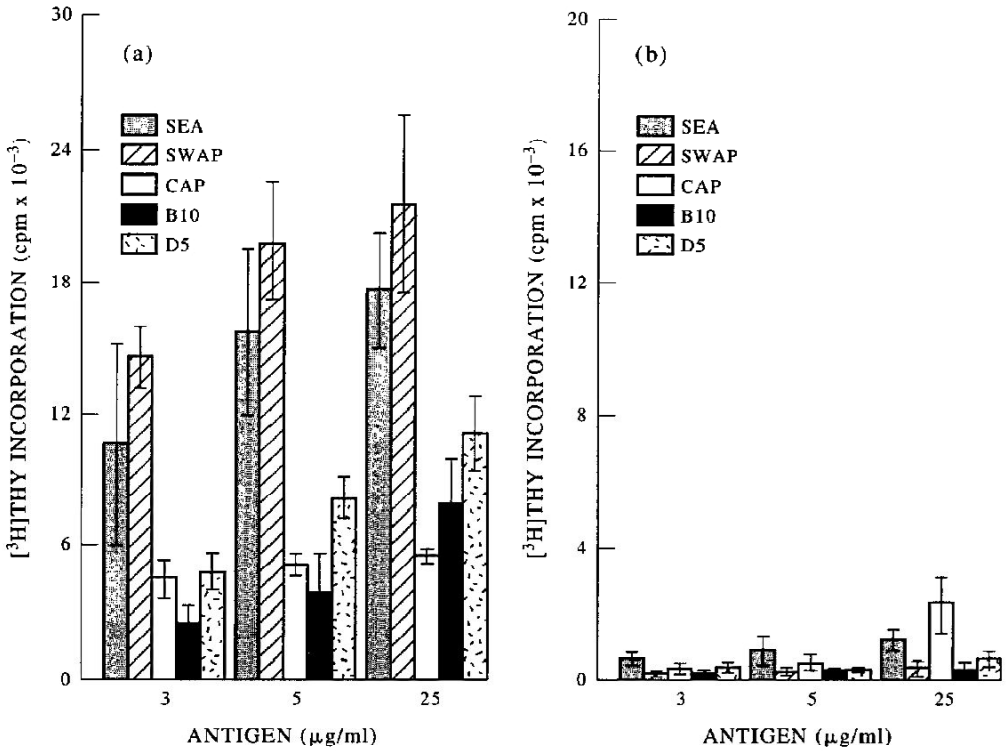

Figure 2 Effect of purified 44.7/56.8 kDa egg antigens on the cell proliferation. PBMC from patients with chronic intestinal schistosomiasis (a) and from normal individuals (b) were incubated with 3, 5 and 25 μg ml^{-1} of SEA (□), SWAP (closely-hatched square), CAP (hatched square) and purified antigens by affinity chromatography with HmAb B10 (shaded square), and HmAb D5 (■). Then, [^{3}H]thymidine incorporation was determined. Data are reported as means SD for (A) N=8 and (B) N=5, in each experimental group. Controls: cells plus medium=0.9×10^3±0.1 c.p.m.; cells plus 25 μg ml^{-1} of PHA=42×10^3±4.5 c.p.m.

Table 3 Protective effect of 44.7/56.8 kDa egg antigens purified by HmAb-B10

Mice treatment	Worm burden[a]	% Protection
None	37±7	—
BSA	35±5	—
44.7/56.8 kDa	20±7*	43

[a]N=8 mice per group performed twice. *P<0.05. Protective effect of 44.7/56.8 egg antigens. Balb/c mice were immunized with 10 μg of antigen in the presence of complete Freund's adjuvant and boosted twice with the same amount of antigen in incomplete Freund's adjuvant. BSA, Bovine serum albumin; % Protection=100×control–experimental/control

Some investigators have provided evidence that IgM antibodies are not required for the resistance to challenge infection induced by vaccination[24]. However, other IgM mouse monoclonal antibodies have been reported to transfer passive protection against challenge infection to *S. mansoni*[25]. Together with the results presented in this paper, it is clear that human IgM monoclonal antibodies are effective in killing schistosomes when directed against particular epitopes and when presented in appropriate concentrations. The epitopes identified by these two monoclonal antibodies were present in cercariae, adult worms and schistosomula, as evidenced by Western blot and immunofluorescence assays[12]. As analyzed by polyclonal or rat monoclonal antibodies, the existence of common or cross-reactive epitopes was indeed identified in the incubation products of adult *S. mansoni* and at the schistosomulum surface[26,27]. The expression of these epitopes is evidently complex, as the SDS–PAGE and two-dimensional gel showed different rates of migration (44.7 kDa and 56.8 kDa) and pI from 7.0 to 7.8, respectively. Recent studies have established protocols of mouse vaccination against *S. mansoni* infection using purified parasite immunogens[7,28,29]. The relevant antigens were identified by means of monoclonal antibodies recognizing the schistosomulum surface and by the passive protection of mice against challenge infection.

In order to support the suggestion that most of the exposed epitopes on the *S. mansoni* life cycle are carbohydrate in nature, the 44.7/56.8 egg antigens were submitted to various treatments which modify carbohydrate residues. Vicinal hydroxyl groups of carbohydrate residues in glycoconjugates are very sensitive to cleavage by low concentrations of periodate at pH 4.5[30,31]. The treatment of SEA with periodate, before incubation with antibodies, did not result in the modification of the antigenic epitopes recognized by these two human monoclonal antibodies. This finding suggests that sugar-containing vicinal hydroxyl group is not involved in B10 and D5 binding. The small increase in B10 and D5 antibodies binding to purified antigens may suggest that the periodate treatment results in additional epitopes being exposed. Similar experiments were carried out with galactose oxidase. This enzyme oxidized the C_6-hydroxyl group of terminal galactose residues to an aldehyde. Incubation of antigens with this enzyme had no significant effect on the binding of B10 and D5. The treatment with TFMS, which has been used for the removal of carbohydrate from biological molecules[19], did not modify antibody binding to these antigens. As these treatments did not demonstrate the presence of carbohydrate in B10 and D5-specific epitopes, carbohydrate epitopes could be a minor contribution to 44.7/56.8 kDa antigenicity. Taken together, these results let

us conclude that the epitopes recognized by HmAb B10 and D5 are apparently polypeptide in nature. This was confirmed by results showed in *Table 2*, which demonstrate that the treatment of antigens with protease K reduced around 73% of antibody binding and treatment with 2-ME inhibited antibody binding by 92%. Interestingly, other studies[32,33] have demonstrated that the majority of exposed epitopes previously discovered on the surface of *S. mansoni* schistosomula and adult worms are carbohydrate in nature.

The role of 44.7/56.8 kDa as protective antigens was supported by the active immunization of mice. Three successive immunizations with these antigens in Freund's adjuvant gave a high level of resistance to a subsequent cercarial challenge. The parasite burden stimulated by liver perfusion was reduced by 43%. These results showed that 44.7/56.8 kDa antigens are potential candidates for immunoprophylaxis against to schistosomiasis. Several groups have demonstrated the potential vaccinating effect of different *S. mansoni* antigens and obtained various levels of protection. Smith and Clegg[28] vaccinated mice with two schistosome surface antigens of 155 and 53 kDa and obtained a level of protection around 30%. Other groups have identified potential antigens for vaccination using indirect methods, particularly by production of monoclonal antibodies associated with protective activity in passive transfer experiments[5,25]. Sher *et al.*[11] identified an immunogen of 97 kDa relevant to the induction of protection in mice. Recently, one such vaccine candidate was described: a 22.6 kDa membrane-associated antigen[8]. This antigen was recognized by IgG antibodies in sera from mice protectively vaccinated with purified adult *S. mansoni* tegumental membranes.

The induction of protective immunity in mice with 44.7/56.8 kDa antigens has been shown to correlate with sensitization of cell-mediated immune responses. Thus, PBMC from infected humans displayed strong proliferation upon stimulation with the same antigens. The association of antigen-specific, cell-mediated immunity with protection is also supported by the evidence presented in previous results[34], in which purified 44.7/56.8 kDa antigens were able to induce proliferation of human T cell clones specific to *S. mansoni* SEA.

This report is the first to describe the purification of *S. mansoni* antigens using human monoclonal antibodies. The purified antigens are associated with protective immunity in mice and may prove to be important as components of an anti-*S. mansoni* vaccine.

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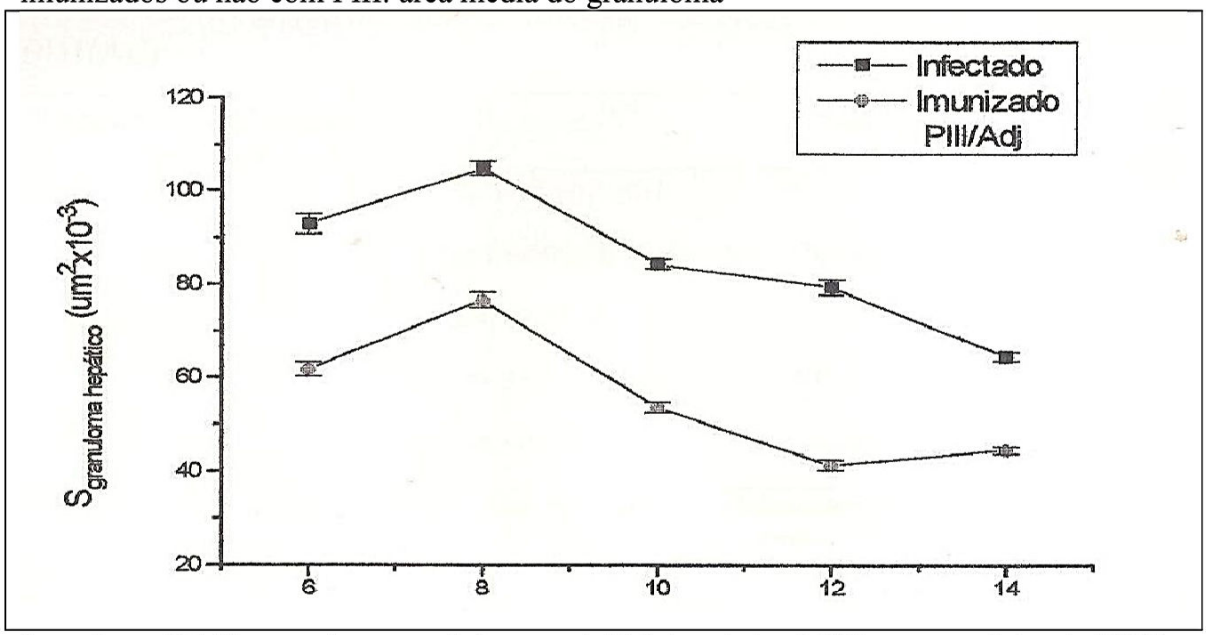
3.2.Resultados complementares

3.2.1. Cinética da formação do granuloma hepático em camundongos imunizados com PIII

Buscando acompanhar o desenvolvimento do granuloma na fase inicial de sua formação, foi feita a infecção de camundongos BALB/c com cercárias viáveis da cepa LE do S. *mansoni*, imunizados ou não com PIII, na presença de adjuvantes (hidróxido de alumínio e *Corinebacterium parvum* - condições experimentais vide Hirsch *et al.*, 1997a). Cortes do fígado fixado em diferentes tempos pós-infecção foram feitos e corados por HE.

O padrão da cinética do granuloma segue o mesmo perfil, independentemente da imunização com PIII (GRÁF. 1), porém a área dos granulomas hepáticos nos camundongos imunizados foi sempre e significativamente menor que aquele dos camundongos não imunizados com PIII para qualquer ponto da curva ($p < 0,05$) (*TAB.* 4), reforçando os resultados obtidos por Hirsch *et al.* (1997b).

GRÁFICO 1: Cinética da formação do granuloma hepático em camundongos BALB/c imunizados ou não com PIII: área média do granuloma



Camundongos BALB/c que receberam, ou não (grupo controle), 3 doses de 1μg de PIII na presença de 1mg de $Al(OH)_3$ e 100μg de *Corinebacterium parvum*, foram infectados com 25 cercárias p.c. e tiveram o fígado colhido, fixado em formalina e corado com HE em diferentes tempos após a infecção (4, 6, 8, 10, 12 e 14 semanas). Os diâmetros, maior e menor, de 20 granulomas contendo apenas um ovo em corte transversal do S. *mansoni*, para cada ponto da curva. Foi feita análise estatística dos dados usando Teste t, com grau de significância de $p < 0,05$. A área do granuloma foi calculada conforme a expressão a seguir e os resultados são mostrados como $S_{média} \pm$ erro padrão:

$$S_{granuloma\ hepático} = 3\ \pi \times \phi_{maior} \times \phi_{menor} / 4$$

TABELA 4: Modulação do granuloma hepático em camundongos BALB/c imunizados com PIII na presença de adjuvantes

Tempo de infecção (semanas) *	$S_{infectado}$**	$S_{imunizado}$**	Redução do granuloma***[t]
6	92.907 ± 42.291	61.772 ± 29.970	33,5%
8	104.809 ± 28.595	76.630 ± 32.901	26,9%
10	84.293 ± 20.251	53.563 ± 20.179	36,5
12	79.353 ± 32.914	41.358 ± 21.728	47,9
14	64.583 ± 18.939	44.565 ± 15098	31,0

* Camundongos imunizados ou não com PIII/Adj foram infectados por via p.c. com 25 cercárias;

** A área dos granulomas hepático - S (μm^2) foi assim: $S_{granuloma\ hepático} = 3\ \pi \times \phi_{maior} \times \phi_{menor} / 4$

*** Redução foi calculada com base na expressão: $[\ (S_{imunizado} - S_{infectado}) \times 100\ ] / S_{infectado}$

t – estatisticamente significativo ($p < 0,05$)

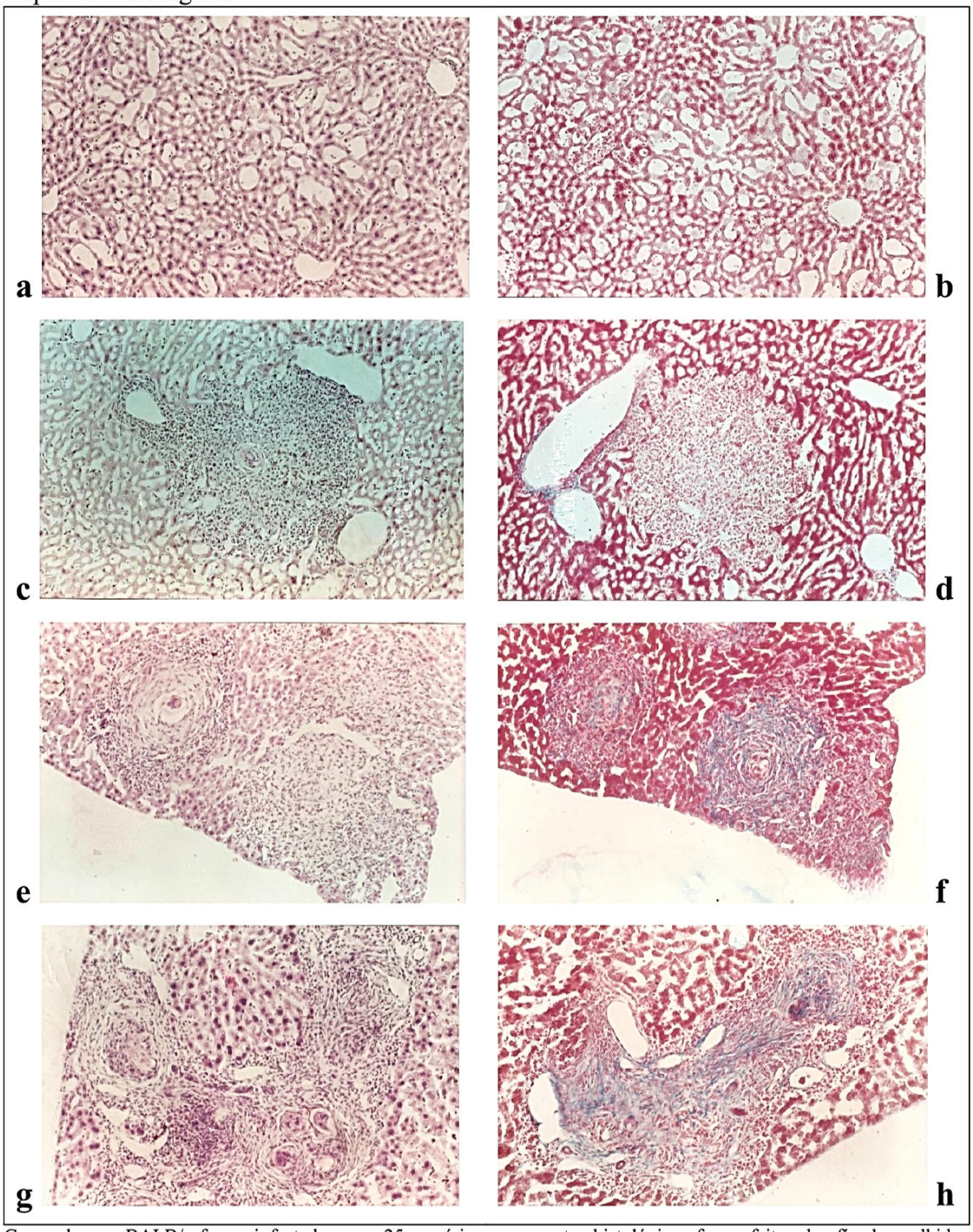
Os cortes histológicos dos fígados dos camundongos, não imunizados com PIII, infectados com cercárias, mostraram que não houve deposição de ovos até a 4ª semana, de modo que o tecido hepático encontrava-se inalterado à coloração de HE (FIG. 1a). Entretanto, a partir da 6ª semana, os granulomas em torno dos ovos retidos no tecido hepático puderam ser visualizados nos cortes corados por HE (FIG. 1c) com evidente aumento da área infiltrada (FIG. 1e e 1g).

3.2.2. Evidenciação da deposição de colágeno no fígado de camundongos imunizados ou não com PIII e infectados com S. *mansoni*

A observação do aspecto histológico dos cortes hepáticos dos fígados corados com HE, nos camundongos imunizados ou não com PIII e infectados por S. *mansoni*, destacou uma área amorfa próxima à deposição dos ovos, de modo mais evidente nas amostras provenientes dos camundongos não imunizados. A fim de investigar a natureza deste achado, foram feitos novos cortes, sincronizados, e corados por HE e por Heidenheim, coloração específica para colágeno. Os cortes corados com Heidenheim (FIG. 1b, 1d, 1 f e 1h) confirmaram que a área amorfa identificada na coloração de HE correspondia mesmo a deposição de colágeno, ou seja, áreas de fibrose (FIG. 1e – corada com HE e FIG. 1f – corada com Heidenheim). Comparando os cortes provenientes de animais imunizados ou

não com PIII, corados com HE, infere-se que esta deposição de colágeno foi menor nos animais imunizados.

FIGURA 1: Cinética da formação do granuloma hepático em camundongos BALB/c: Aspectos histológicos



Camundongos BALB/c foram infectados com 25 cercárias s.c. e cortes histológicos foram feitos dos fígados colhidos com 4 (**a** e **b**), 6 (**c** e **d**), 8 (**e** e **f**) e 10 (**g** e **h**) semanas e em seguida fixados e corados por HE (**a**, **c**, **e** e **g**) ou Heidenheim (**b**, **d**, **e** e **h**). Aumento de 250x.

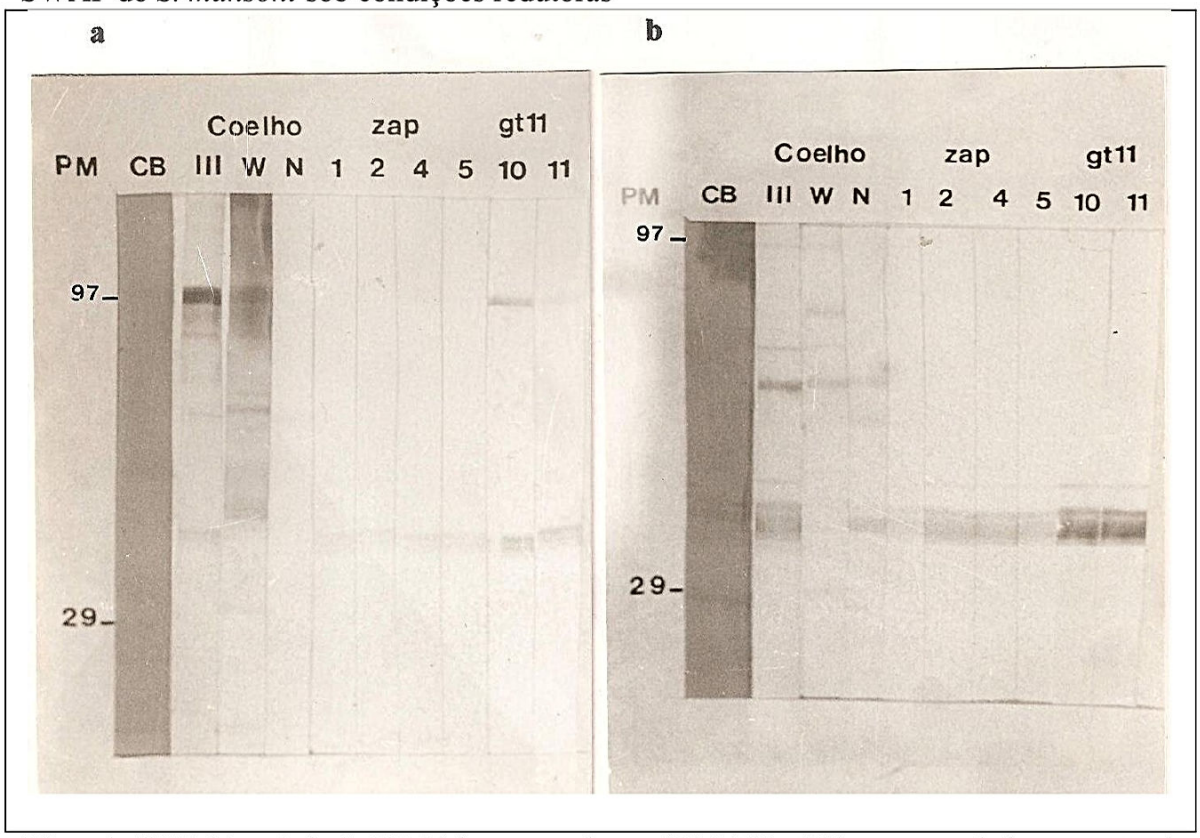
3.2.3. Isolamento de clones de cDNA de vermes adultos do S. *mansoni* em bibliotecas contidas em vetor λzap

Buscando avançar na caracterização dos componentes de PIII, foi feita a pesquisa de clones de cDNA de verme adulto do S. *mansoni* em uma biblioteca contida em λzap. Após analisar cerca de 240.000 pfu usando o soro de coelho imunizado com PIII, o mesmo usado para a pesquisa em uma biblioteca λgt11 (HIRSCH *et al.*, 1997b), a partir da qual foram selecionados quatro clones, designados SmIII.1; SmIII.2, SmIII4 e SmIII.5.

Visando identificar as características dos produtos recombinantes contidos nos fagos selecionados, os Ac específicos contidos no soro de coelho anti-PIII, para cada clone, foram isolados (vide HIRSCH *et al.*, 1997b, para detalhes da eluição dos Ac usando fagos de expressão) e usados em ensaios de imunoblotagem (Western Blot), sobre tiras de nitrocelulose contendo os componentes da fração PIII e de SWAP, devidamente separados por eletroforese (SDS-PAGE) em gel de poliacrilamida a 10% sob condições redutoras (FIG. 2) ou não (FIG. 3). Para comparação, também são mostrados os dados para os clones λgt11.

Foi possível observar que os Ac reativos para os clones λzap foram capazes de reconhecer componentes de mesmo peso molecular, tanto em SWAP como em PIII reduzidos, e de modo semelhante que os Ac para os clones λgt11 (FIG. 2).

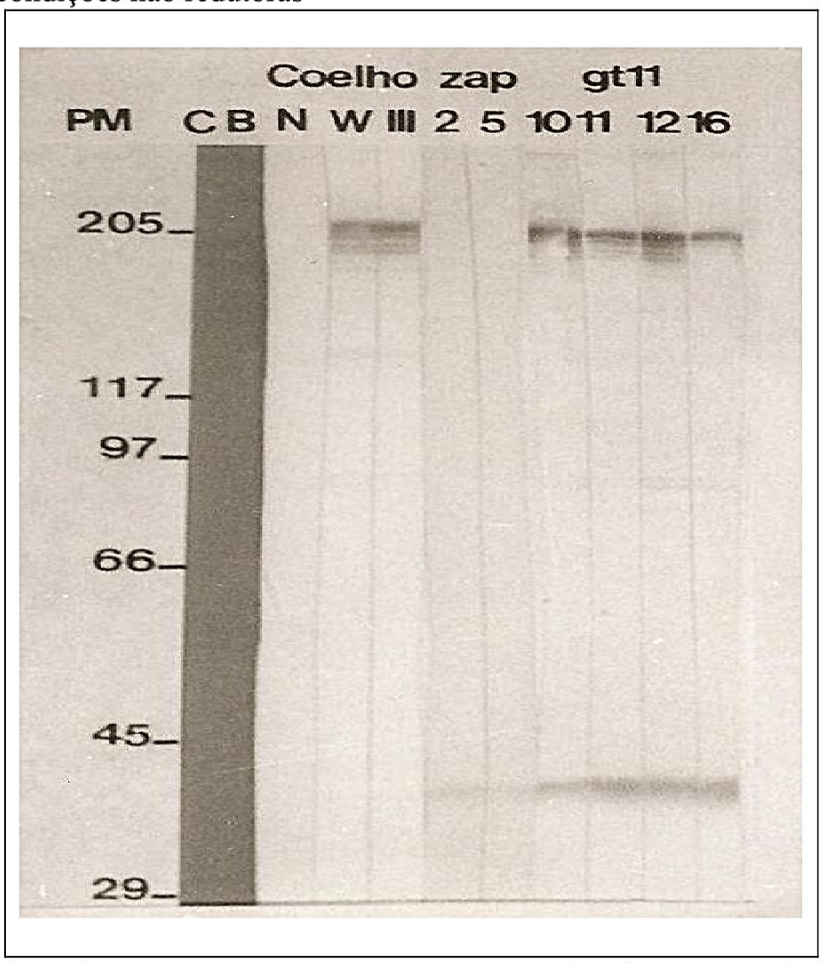
FIGURA 2: Reatividade de Ac clone-específicos a componentes antigênicos de PIII e de SWAP do *S. mansoni* sob condições redutoras



250 mg de SWAP (**a**) ou da fração PIII (**b**) foram separadas em SDS-PAGE a 10% na presença de β-mercaptoetanol e depois transferidos para nitrocelulose. Tiras do material transferido foram incubadas com soro de coelho normal, anti-PIII diluídos 1:50 e anti-SWAP, 1:1000. Os Ac clone-específicos foram diluídos 1:2. Soro revelador foi de cabra anti-Ig de coelho conjugada à peroxidase (1:5.000) ou fosfatase alcalina (1:10.000). **Legenda**: CB – transferência corada com Comassie Blue; soros reveladores: **III** – coelho total anti-PIII, **W** – coelho total anti-SWAP; **N** – coelho não imunizado, normal, **1** – Ac clone λzap SmIII.1 específico; **2** – Ac clone λzap SmIII.2 específico; **4** – Ac clone λzap SmIII.4 específico; **5** – Ac clone λzap SmIII.5 específico; **10** – Ac clone λgt11 SmIII.10 específico; **11** – Ac clone λgt11SmIII.11 específico.

Quando a fração PIII foi tratada com agente redutor (β-mercaptoetanol) apenas um componente da amostra foi reativo com os Ac específicos para os clones λzap, enquanto que os Ac para os clones λgt11 foram capazes de ligar também componentes de maior peso molecular (FIG. 3).

FIGURA 3: Reatividade de Ac clone-específicos a componentes antigênicos de PIII do *S. mansoni* sob condições não redutoras



250 mg de da fração PIII foram separadas em SDS-PAGE a 10% na ausência de β-mercaptoetanol e depois transferidos para nitrocelulose. Tiras do material transferido foram incubadas com soro de coelho normal, anti-PIII diluídos 1:50 e anti-SWAP, 1:1000. Os Ac clone-específicos foram diluídos 1:2. Soro revelador foi de cabra anti-Ig de coelho conjugada à peroxidase (1:5.000) ou fosfatase alcalina (1:10.000). **Legenda**: CB – transferência corada com Comassie Blue; soros reveladores: **III** – coelho total anti-PIII, **W** – coelho total anti-SWAP, **N** – coelho não imunizado, normal, **2** – Ac clone λzap SmIII.2 específico, **5** – Ac clone λzap SmIII.5 específico; **10** – Ac clone λgt11 SmIII.10 específico, **11** – Ac clone λgt11SmIII.11 específico, **16** – Ac clone λgt11SmIII.16 específico.

Estes resultados sugerem que os quatro clones λzap devem codificar a mesma proteína, ou pelo menos proteínas de mesmo peso molecular que possuem epitopos comuns com aqueles codificados pelos clones λgt11.

4. CONSIDERAÇÕES FINAIS

Nas últimas décadas, vários trabalhos foram feitos visando identificar componentes do parasito que pudessem controlar, se não a infecção pelo *S. mansoni*, pelo menos o desenvolvimento de fêmeas adultas, dificultando a oviposição e com isso reduzindo a morbidade na esquistossomose (WHO, 1991). Desta maneira, foram obtidos Ag parcialmente purificados ou frações antigênicas com efeitos bastante diversificados. As estratégias incluem desde métodos convencionais de filtração, até cromatografias de afinidade ou de troca iônica de alta resolução (FPLC e HPLC). Recentemente, o uso de tecnologia de DNA recombinante vem fornecendo Ag purificados em maior quantidade. Em nosso trabalho utilizando cromatografia de afinidade e de troca iônica, fomos capazes de:

1. Identificar Ag de SEA através de reatividade com AcMH, obtidos a partir de pacientes esquistossomóticos forma crônico-intestinal (PCI), capazes de induzir proteção em infecção experimental em camundongos; e
2. Obter uma fração aniônica de SWAP reativa com Ac e células de PCI que, além de proteger em modelos experimentais, também se mostrou relevante na modulação de granulomas *in vivo*.

Hirsch e Goes (1996) já haviam caracterizado a reatividade de células mononucleares do sangue periférico (MNSP) e de soro de PCI a pelo menos cinco das seis frações de SWAP obtidas em cromatografia de troca aniônica usando Q-Sepharose. A reatividade do soro de oito PCI a fração PIII apresentou valor intermediário quando comparado às demais frações, mas a reatividade celular dos MNSP destes mesmos pacientes frente à Fração PIII em ensaio de proliferação celular apresentou níveis elevados. Ag de vermes adultos geralmente têm sido associados à imunidade protetora, entretanto alguns trabalhos têm procurado avaliar um possível papel modulador da reação granulomatosa (DOUGHTY; PHILLIPS, 1982a; DOUGHTY *et al.*, 1987; BOULANGER *et al.*, 1991), já que os vermes adultos se alojam muito próximos aos sítio de deposição dos ovos, onde ocorre a reação granulomatosa. Além disso, grande quantidade de Ag do verme é constantemente liberada neste microambiente. Pensando nisto, o ensaio de granuloma *in vitro* foi usado para evidenciar o papel do Ag do verme adulto nesta reação. A reatividade granulomatosa *in vitro* medida através da determinação do índice de granuloma que é

obtido pela avaliação da reatividade/aderência das células dos PCI a pérolas de poliacrilamida recobertas com Ag das diferentes frações. Neste ensaio, os Ag da fração PIII não induziram formação do granuloma *in vitro* (HIRSCH; GOES, 1996). Estes resultados, tomados juntos, nos sugeriram que os Ag da fração PIII teriam participação na modulação do granuloma observada nestes pacientes. Doughty e Phillips (1982b) já haviam mostrado a participação de Ag do verme adulto na modulação da reatividade granulomatosa aos ovos do parasito em ensaios *in vitro*.

Muito já foi esclarecido com respeito à transferência de imunidade e o estabelecimento da imunidade protetora, através de estudos da reatividade de Ac de PCI ou camundongos na infecção experimental. AcMH produzidos a partir de PCI estimulados com SEA (GOES *et al.*, 1989) foram usados em cromatografia de afinidade para purificar os Ag por eles reconhecidos. Foi comprovada a atividade anti-esquistossômulo *in vitro*, assim como através de ensaios de transferência passiva deste AcMH. Prosseguindo, foi demostrado que os Ag reconhecidos por estes AcMH, purificados em cromatografia de afinidade, foram capazes de proteger camundongos contra infecção após imunização (HIRSCH *et al.*, 1997c). Por sua vez, Ag de ovo são geralmente associados à reatividade granulomatosa, sua ativação e modulação, e poucos são os trabalhos que avaliam possível indução de imunidade protetora. Os resultados aqui mostrados mostram uma proteção de 43%, reforçando a validade do desenho de uma vacina múltipla.

A fim de testar a habilidade de PIII induzir imunidade protetora e modulação do granuloma *in vivo*, camundongos foram imunizados e desafiados com cercárias viáveis do *S. mansoni*. Nossos resultados mostraram que PIII induziu níveis relevantes de proteção e reduziu o tamanho do granuloma hepático e pulmonar (HIRSCH *et al.*, 1997a), confirmando a ideia obtida a partir da estimulação de células humanas (HIRSCH; GOES, 1996).

Usando bibliotecas de cDNA de vermes adultos do S. *mansoni* e soro de coelho imunizado com fração PIII, foram isolados clones de vetores que codificam produtos reconhecidos pro este soro. A análise da sequência de DNA mostrou que os 4 clones, isolados a partir da biblioteca contida em fago λgt11, codificavam um produto com 100% de homologia com a paramiosina (HIRSCH *et al.*, 1997b), sugerindo que esta proteína seja o principal componente da fração PIII.

Nossos resultados apontam no sentido de complexas interações entre Ac, Ag e epitopos de diferentes estágios do parasito, verme adulto e ovo, para o desenvolvimento tanto da imunidade protetora como para a modulação da hipersensibilidade granulomatosa aos ovos do parasito. Muitos mecanismos podem estar envolvidos, por exemplo:

1. Ag de PIII estimulam LT, que participam diretamente da modulação do granuloma e regulação da produção de Ac;
2. Ag de PIII poderiam, em PCI, estimular a produção de Ac capazes de mobilizar uma rede interações idiotípicas com linfócitos T e B, que modulam a resposta imunológica aos Ag do ovo e ao verme, permitindo uma convivência menos danosa, ao mesmo tempo em que controla contra reinfeções;
3. Ag presentes no ovo e no verme adulto participam desta rede conectando Ac e linfócitos em uma resposta harmoniosa, que pode envolver a paramiosina, constituinte principal da fração PIII, e os componentes de 44,8/56,7k, isolados a partir de Ag de ovo com AcMH anti-SEA.

Citocinas desempenham um papel crucial neste processo e ensaios estão sendo executados para se avaliar o perfil de secreção de citocinas induzido pela fração PIII em células de pacientes esquistossomóticos. Os ensaios de imunização experimental em camundongos com PIII mostraram que seus componentes induzem granulomas menores (HIRSCH *et al.*, 1997a) e menor deposição de colágeno (resultados complementares). É provável que o estímulo *in vitro* com a fração PIII induza a produção de IFN-γ e IL-2 que, juntos, têm um efeito anti-inflamatório (LAMMIE *et al.*, 1986; LUKACS; BOROS, 1993) e antifibrosante (CHEEVER *et al.*, 1992), o que explicaria os resultados encontrados. Ambos os efeitos seriam sinergizados pela produção de IL-12 por macrófagos do granuloma (WYNN *et al.*, 1994 e 1995).

O presente trabalho não se fecha em si mesmo, mas abre novas perspectivas quanto á intensa correlação entre os Ag provenientes dos diferentes estágios do parasito e os mecanismos imunológicos envolvidos na indução de imunidade protetora e na modulação da ração granulomatosa. Ag de ovos e d vermes adultos parecem ter papel importantíssimo em ambos os processos e muito ainda há que ser elucidado.

5. CONCLUSÃO

Os antígenos 44,8/56,7k, de SEA, e a fração PIII, de SWAP, do *Schistosoma mansoni*, relacionados à reatividade imunológica de pacientes esquistossomóticos crônico-intestinais, foram capazes de induzir imunidade protetora e diminuir a resposta granulomatosa aos ovos do parasito no modelo experimental *in vivo*, demonstrando a intrincada rede de interações antigênicas que caracteriza a fisiopatogenia da esquistossomose.

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