(51) Int Cl⁵:
A61K 39/00

REPÚBLICA FEDERATIVA DO BRASIL
Ministério da Indústria, do Comércio e do Turismo
Instituto Nacional da Propriedade Industrial

(11) (21) PI 9305075-5 A

(22) Data de Depósito: 16/12/93

(43) Data de Publicação: 08/08/95 (RPI 1296)

(54) Título: Antígeno para conferir imunidade protetora contra infecções helmínticas em humanos e animais, e processo de vacinação para aplicação na imunoprofilaxia de doenças helmintológicas de interesse veterinário e médico

(57) Resumo Essa invenção se refere de forma geral a material antígeno derivado de helmintos capaz de induzir eficaz e duradoura proteção contra parasitas, em particular a antígenos que servem para intermediar imunidade protetora contra helmintos.

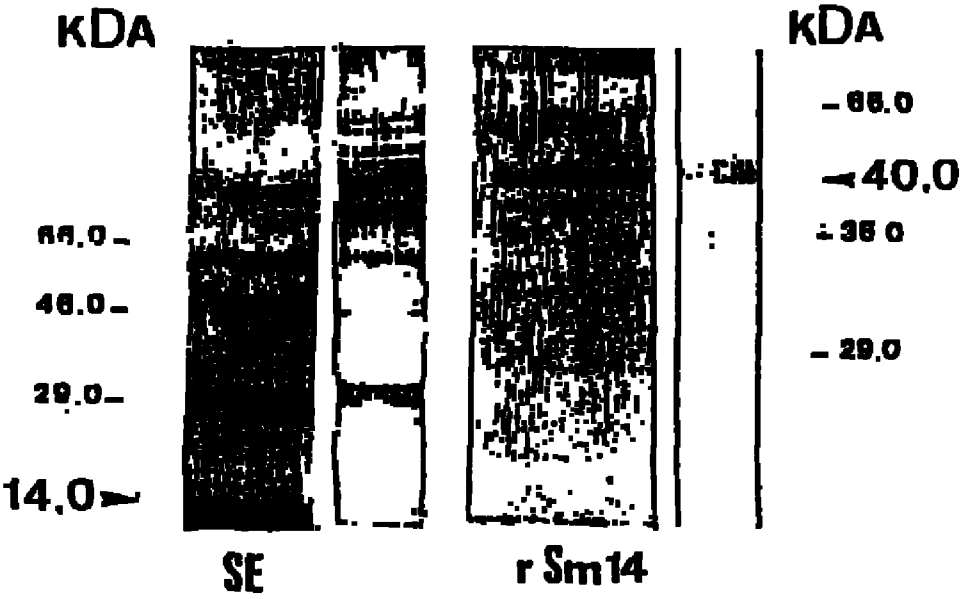
(71) Depositante(s): Fundação Oswaldo Cruz (BR/RJ)

(72) Inventor(es): Miriam Tendler; Nathale Katz; Andrew John Simpson

(74) Procurador: Franco, Sharing, Barbosa e Novaes

Fig 1

GEL DA PREPARAÇÃO FINAL DO ANTÍGENO
(purificação de rSm 14) EM COMPARAÇÃO AO SE



Relatório Descritivo da Patente de Invenção: "ANTÍGENO PARA CONFERIR IMUNIDADE PROTETORA CONTRA INFECÇÕES HELMÍNTICAS EM HUMANOS E ANIMAIS, E, PROCESSO DE VACINAÇÃO PARA APLICAÇÃO NA IMUNOPROFILAXIA DE DOENÇAS HELMINTOLÓGICAS DE INTERESSE VETERINÁRIO E MÉDICO"

Fundamentos da Invenção

Essa invenção se refere de forma geral a material antígeno derivado de helmintos capaz de induzir efetiva e duradoura proteção contra parasitas, em particular a antígenos que servem para intermediar imunidade protetora contra helmintos.

Dentre os helmintos os trematódeos digenéticos ou "vermes", compreendem cerca de 100 famílias. A maioria são parasitas comparativamente inofensivos localizados no intestino e outros órgãos de vertebrados e, consequentemente tem recebido atenção mínima dos parasitologistas. Aqueles trematódeos que causam sérias doenças no homem são os "vermes" de sangue ou schistosomas e os "vermes" de fígado e pulmão os quais são parasitas muito importantes que infectam os animais.

Fasciola a mais importante dos "vermes" de fígado é o parasita encontrado principalmente em ruminantes domésticos e é responsável por sérias perdas econômicas através do mundo (gado bovino, ovelha, cabra).

A característica principal da doença e que é responsável pela patologia, morbidez e mortalidade dos animais mencionados, é a destruição do tecido do fígado hospedeiro e dano aos canais da bilis. A morbidez é mais alta em animais novos que são especialmente afetados e se tornam magros e morrem. A Fasciola pode também parasitar o homem, quando dada a oportunidade, e é mais frequente em Cuba e países da América Latina. Entretanto, o "verme" de fígado do homem é outro parasita, denominado Clonorchis sinensis que é muito difundido na China, Japão, Coréia, Vietnam e Índia. A patologia é basicamente causada por afinamento das paredes dos canais de bilis, em muitos casos causa cirrose do fígado e morte.

A Fasciola e Clonorchis entram passivamente como metacercariae ingerida com a alimentação (vegetação e peixe cru para Fasciola e Clonorchis, respectivamente), porém suas rotas de migração no corpo do hospedeiro vertebrado para os canais de bilis são diferentes.

Enquanto Clonorchis corre pela ramificação biliar a partir do intestino através dos canais de Vater, a Fasciola migra através da cavidade abdominal, pentrando sucessivamente na parede do intestino e parenquima do fígado, causando sérios danos aos tecidos do hospedeiro.

Em relação à Fasciolose em animais domésticos, existem resultados conflitantes e pouca evidência para sugerir que a ovelha ou cabra adquirem imunidade contra Fasciola hepatica (Sinclair, 1967) após a imunização com extratos crus.

Existem também evidências para mostrar que a infecção pode persistir por pelo menos 11 anos em ovelhas experimentalmente infectadas (Durbin, 1952). Pode também ser relatado que ocorre muito pouca ou nenhuma reação do hospedeiro contra o parasita: assim a sobrevivência da ovelha dependerá inteiramente do número de metacercariae ingeridas (Boray, 1969). O gado bovino é considerado ser mais resistente: Fasciola hepatica geralmente permanece nesse

hospedeiro por 9 - 12 meses, porém é o gado mais novo que apresenta a fasciolose clínica mais séria.

Foram feitas diversas tentativas para identificar antígenos imunoprofiláticos que poderiam prover boa base para desenvolver uma vacina eficiente contra a Fasciolose. Basicamente, tem sido perseguido por diversos cientistas duas estratégias experimentais independentes as quais se baseiam em: 1) imunidade induzida por vacinas vivas irradiadas, e 2) imunidade induzida por vacinas não - vivas.

Todavia, foram publicados poucas tentativas sobre resistência adquirida à Fasciola hepatica em novilhos usando extratos de trematódeo (Ross 1967; Hall e Lang, 1978; Hillyer, 1979), e além disso os dados relatados são conflitantes.

A imunidade induzida por vacinas vivas irradiadas tem também mostrado resultados frustrantes em experimentos realizados em camundongos, coelhos ou ovelha (Campbell e outros 1978), Hughes 1963); já que não há evidência de imunidade desenvolvida nesses animais, seguida da administração de metacercariae irradiada.

Adicionalmente, os experimentos com diferentes extratos ou produtos excretor/secretor a partir de trematodes de estágios adulto não foram imunogênicos, mostrando que os animais vacinados apresentavam baixa proteção e lesões patológicas no parenquima do fígado.

Como refletido no estado da técnica, era esperado que o gado bovino respondesse melhor em relação as vacinas não vivas, porém era duvidoso se prognósticos similares poderiam ser feitos em ovelhas, com base somente na medíocre proteção induzida por um número de antígenos diferentes em animais experimentais.

A indução de imunidade protetora contra Fasciola hepatica por meio de infecção de ovelha com Cysticercus tenuicollis, que é o estágio metacestódeo da

tênia de cachorro Taenia hysdatigena, produziu proteção parcial contra Fasciola hepatica, porém Hughes e outros (1978), todavia, não confirmam esse resultado. Outros experimentos foram também incapazes de induzir proteção contra Fasciola hepatica em animais experimentais com esse verme (tênia).

Os camundongos infectados com vermes adultos bissexuais de S. mansoni estatisticamente desenvolveram significante resistência à Fasciola hepatica e infecções simultâneas com ambos os parasitas resultaram em um número reduzido de schistosomas existentes e reduziu a produção de ovos de schistosoma por verme (Christensen e outros, 1978). Os novilhos infectados com S. bovis também mostraram muita resistência à Fasciola hepatica e dano ao tecido do fígado, menos pronunciado (Sirag e outros, 1981).

Pelley e Hillyer, 1978; Hillyer e de Atica, 1980, relataram antígenos comuns entre Fasciola hepatica e Sohistosoma mansoni encontrados nos ovos de Sohistosoma. Outra descoberta que indica imunidade de reação cruzada é a ocorrência de reações falso positivas nas áreas onde ambos os parasitas são endêmicos, Hillyer, 1985 e Hillyer e outros, 1987, demonstraram também que uma mistura de antígenos derivados de Fasciola hepatica pode conferir proteção contra infecções subsequentes com Fasciola hepatica e S. mansoni.

A esquistosomose ou Bilharzia é uma antiga doença proveniente da água, relatada pelos Egípcios a 4000 anos atrás e hoje é um problema de saúde pública em todo o mundo. É estimado que essa doença aflige mais de 200 milhões de pessoas nas áreas urbanas e nos perímetros urbanos do Terceiro Mundo. Os três principais schistosomas que infectam o homem são transmitidos pela lesma de água fresca e a larva nadando livre, chamada cercarine, a qual é desprendida na água, e é capaz de penetrar diretamente na pele do hospedeiro. Após a migração a partir da derme através dos pulmões para o sistema hepático, os schistosomas vão viver nas pequenas veias mesentérica ou pélvica, onde cada fêmea deposita até 100 ovos por

dia na corrente sanguínea. A reação imune do hospedeiro aqueles ovos os quais se alojam nos tecidos é largamente responsável pela debilidade crônica e frequentemente a doença fatal. A extensão dos esquemas de irrigação, a construção de represas e a concentração de populações humanas são hoje, fatores contribuidores para aumentar a distribuição e intensidade da infecção de Schistosoma. O controle de larvas e a quimioterapia são os elementos principais, mas através de métodos não satisfatórios de controle. Uma vacina eficiente seria um marco para ajudar consideravelmente as tentativas de erradicar a doença.

Uma variedade de espécies hospedeiras pode desenvolver resistência parcial ao S. mansoni seguindo infecção anterior ou imunização com cercariae atenuada por radiação (Smithers & Doenhoff, 1982). O estado da técnica com relação a possibilidade experimental de imunização contra infecção de S. mansoni (Clegg & Smith, 1978) foi substituído pelo atual entusiamo para a possibilidade de produzir uma vacina definitiva e efetiva contra esse parasita com vacinas mortas (Tendler, 1987). Entretanto, a principal limitação permanece no grau incompleto de proteção alcançado em animais na maioria dos experimentos com antígenos de parasitas purificados e quimicamente definidos. Como descrito por diversos autores e revisto por Smithers, 1982 houve um consenso na necessidade de aumentar o nível de proteção induzida por imunoprofilaxia experimental. Todavia, o estabelecimento de um modelo de animal para o desenvolvimento de uma vacina eficiente contra a esquistesomose tem sido muito difícil de alcançar. O progresso depende da identificação e purificação de moléculas antigênicas altamente efetivas que poderiam intermediar a imunidade protetora, Schistosoma mansoni: Protective Antigens. M. Tendler - Mem. Inst. Oswaldo Cruz RJ, vol. 82, suppl IV 125 - 128, 1987.

Em estudos anteriores na busca de antígenos que intermediam a imunidade protetora contra esquistosomose, relata-se o uso de um coquetel de componentes de

Schistosomas (chamado SE) liberados durante a incubação de vermes adultos de S. mansoni vivos e frescamente perfusados em solução salina de fosfato tamponada (Tendler & Scapin, 1979; Köhn e outros, 1979). Focalizando a atenção para alcançar a proteção contra infecção por cercariae usando como uma vacina, foi projetado um modelo experimental, em dois diferentes hospedeiros de animal, o camundongo suíço e coelhos da Nova Zelândia, conhecidos serem completamente susceptíveis e parcialmente resistentes a infecção por S. mansoni, respectivamente.

No modelo de S. mansoni de coelhos da N. Zelândia, foi possível estabelecer um padrão confiável de infeções percutâneas, com cargas de vermes adultos homogêneas, em termos de números e tamanhos de parasitas e razões macho/fêmea, por um longo período após a infecção (Tendler, 1982, 1985, 1986). As evidências recentes sugerem que o uso dos coelhos como hospedeiro experimental para S. mansoni pode representar um novo modelo de imunidade para a doença (Almeida e outros, 1987).

Os experimentos de imunização realizados em coelhos com a mistura de SE, resultou em altos níveis de proteção no desafio (Scapin e outros, 1980; Tendler, 1980; Tendler e outros 1982) (90% de redução de carga parasitária em animais imunizados comparados aos controles normais equilibrados em sexo e idade, quando desafiados simultaneamente com o mesmo número e soma de cercariae ativa da cepa - LE de S. mansoni). Os camundongos suiços imunizados com SE, também tem mostrado serem significativamente protegidos contra o desafio com cercariae normal e completamente resistente à infecção letal (Tendler, 1986). Para medir a resistência, os animais vacinados e desafiados, e os controles em paralelo são submetidos a perfusão hepática e mesentérica para determinação de cargas de parasita adulto. O grau de proteção é calculado pela diferença em número de parasitas recuperados a partir do controle versus os animais vacinados (Tendler e outros, 1982).

À luz das evidências in vitro de que anticorpos formados contra diferentes estágios de desenvolvimento dos parasitas são efetivos em eosinofil ou sistemas de citotoxidade (Grzych e outros, 1982; Smith e outros, 1982), a caracterização de antígenos reconhecidos pelos soros a partir de hospedeiros imunes, é usada para identificar moléculas de antígenos no que concerne a imunidade protetora (Bickle e outros, 1986; Horowitz & Arnon, 1985). Os experimentos de "western blot" foram empreendidos para analisar a resposta de anti-corpo de coelhos vacinados com SE. Na investigação de antígenos SE com um Tabela de anti-soros derivados de coelhos imunizados pelo mesmo esquema (SE - FCA), os autores foram capazes de demonstrar em ensaios de imunoblot, dois padrões distintos de reconhecimento de antígenos de SE nesses indivíduos. De forma interessante, alguns antígenos de SE foram restritamente reconhecidos somente pelos anti-soros de coelhos quase completamente protegidos. Essa descoberta permitiu aos autores identificar dois sub-Tabelas de antígenos no SE: um comum a todos os anti-soros individuais de coelhos, e um segundo sub-Tabela restrito a animais altamente protegidos. Esses dois padrões foram respectivamente nomeados padrões de Baixa Proteção e Alta proteção e usados como anti-corpos "diferenciais". Tirando vantagem desses dois padrões de reconhecimento de componentes de SE por anti-corpos policlonais a partir de coelho que responderam "diferencialmente" ao mesmo esquema de imunização, (provavelmente por conta da variação individual, esperada de ocorrer em populações de camundongos suíços nascidos do intercruzamento de linhagens não relacionadas), a estratégia de detalhar cDNA de arquivos com aqueles soros foi aplicada. Com a restrição de entendimento incompleto de mecanismos críticos de resposta protetora em ambos esquistosomose experimental e humana, os procedimentos de detalhamento adotados por outros frequentemente envolveu o uso de soros de indivíduos (humanos) infectados (indivíduos "putativo", imune ou susceptível de áreas endêmicas (Carter of Colley, 1986) ou soros selecionados de

monoclonal ou policlonal a partir de animais imunizados (Lanar e outros, 1986; Balloul e outros, 1987), que são direcionados contra diversos antígenos não caracterizados.

Nas tentativas iniciais em direção a clonagem molecular de componentes de SE potencialmente ativos dois cDNA de arquivo a partir de vermes adultos de S. mansoni e S. japonicum construidos pelos Drs. Klinkert Universidade de Heidelberg e Donnelson/Henkle, Universidade de Lowa, respectivamente, foram detalhados com filtros duplicados por detalhamento diferencial. Poderia ser traçado um paralelo com os resultados dos imunoblots no que foram detectados dois Tabelas diferentes de clones, o que presumidamente corresponde à diferença no reconhecimento por soros anti - SE de coelhos susceptíveis e resistentes. Em experimentos adicionais visando a identificação dos componentes do SE, foram comparados em imunoblots, soros anti - SE policlonal de coelho (Alta e Baixa proteção) com anti-soro de coelho para purificar paramiosina de schistosoma. Essa proteina é uma molécula recentemente definida, parcialmente protetora contra infecção desafiada de S. mansoni em camundongos suíços nascidos do intercruzamento de linhagens relacionadas, mostrando ser sensível a degradação proteolítica à dois principais produtos partidos de Mr (x10-3) 95 e 78 (Pearce e ouros, 1986).

O complexo de 97/95/78 kD foi reconhecido pelas proteções Alta e Baixa de soros anti - SE e soro anti - paramiosina mono específico. A proteção "Alta" de soros anti - SE reconhecem além da paramiosina, outros polipeptidios que permaneceram para que fossem caracterizados e avaliados de acordo com suas atividades produtoras e sua ação imunológica. A descoberta da paramiosina como um componente de SE reforça estudos anteriores indiretos de imunofluorescência, conduzidos em porções de Schistosomas adultos com soro anti - SE de coelhos, o qual reagia com ovos na superfície parasitária e entre as camadas musculares

(Mendonça et al., 1987), de uma forma similar conforme demonstrado na paramiosina (Pearce et al., 1986). Esta descoberta também comparou os resultados obtidos por arquivos de imunoproteção de cDNA conforme o anteriormente mencionado. Novamente clones de paramyosim comuns foram isolados com ambos soros de anti - paramiosina e anti - SE, sendo os extra clones identificados somente no último soro de coelho (Alta proteção). Dentre outros componentes SE de baixo peso molecular o 31/32 kD idêntico, descrito como um possível candidato para diagnosticar esquistosomose (Klinkert et al., 1987) foi também identificado e recentemente considerado como uma prótese localizado no intestino de um schistosoma. Estes antígens e outros que foram identificados no extrato salino demonstraram uma proteção muito baixa quando testados.

A incubação de schistosomas recentemente perfusados num meio quimicamente definido (PBS) foi direcionada na extração de uma liberação precoce de antígens de vermes adultos vivos (especialmente produtos excretórios/secretórios e componentes tegumentais). Essa estratégia foi adotada em decorrência de uma tentativa frustrada a fim de induzir uma resistência estável contra uma infecção esquistossomática com diferentes extratos crus de S. mansoni, que teoricamente poderia ser exaurido de antígens de relevante função. Essa premissa foi basicamente influenciada pelo tipo de processo de extração comumente utilizado que originou do uso de parasitas mortos. Na realidade utilizando o SE emulsificado em FCA (como adjuvante preferencial) e administrado por vias subcutâneas/intradermal, foi obtido em dois animais hospedeiros uma proteção contra a infecção S. mansoni com prazo de duração alto e longo. O que foi razoável pelo uso de uma cobaia coelho, um tanto raro como proteção experimental foi alcançar um trajeto potencialmente preventivo e antígens separados num hospedeiro parcialmente resistente (a ser testado em hospedeiros sensíveis) os quais poderiam portanto "aumentar" a reação imunológica e os

mecanismos causadores da morte parasitária, tendo em vista que os coelhos por serem conhecidos como fortes produtores de anti - corpos foram considerados um instrumento de muita importância neste assunto.

Estudos da reação imunológica induzida em animais vacinados, objetivando a identificação de componentes protetores do SE de relevância funcional, localização e mecanismos de morte parasitária e marcadores protetores têm sido o enfoque dos esforços recentemente, muito embora pouca informação, tanto sobre a composição molecular do SE quanto sobre a identificação e isolamento de seus componentes protetores seja disponível até o presente momento.

A Patente US 4 396 000 publicada em 02.08.1983 em nome de Luigi Messineo & Mauro Scarpin (tal patente encontra-se extinta segundo o certificado de reexame 461st B1 4 396 000 publicado em 11.02.1986) descreve um extrato de vermes adultos de Schistosoma mansoni obtido através de incubação em uma solução tamponada 0.15M de cloreto de sódio - fosfatado de sódio (PH 5.8) continha carboidratos proteicos e ácido nucleico e/ou subprodutos do último componente e transformou-se em 4 frações principais através de cromatografia gelatinosa em colunas Sephadex G-100 e G-200. Testes de imunodifusão com soro de extrato anti - total de coelho revelaram 3 linhas de precipitação correspondentes às frações I e II e uma correspondente a III ou IV. Os coelhos que foram imunizados por esse extrato total são considerados ser totalmente ou parcialmente pelo menos 77% resistentes a uma infecção desafio. O material antigênico de extrato salino é uma vacina eficiente no tratamento e imunização de esquistosomose e outras infecções causada por Schistosoma.

A ação oficial acima mencionada foi baseada principalmente em dois artigos dos inventores e foram usados aqui como o princípio da presente invenção dentre os inúmeros arquivos que correspondem aos antecedentes da presente invenção, a mais recente informação foi a clonagem e o sequenciamento de um

componente derivado do SE, indicado como Sm-14.

O estudo mais recentemente publicado é o A 14-KDa Schistosoma mansoni "Polypeptide is Homologous to a gene family of fatty Acid Binding Proteins - Jornal de Química Biológica - vol. 266 No. 13, publicado em 05 de Maio, pp. 8477 - 8454, 1991 D. Moser, M. Tendler, G. Griffiths, e Mo-Quen Klinkert". Este estudo descreve o sequenciamento do gene e a demonstração da atividade funcional do Sm-14 como uma proteína que aglutina lipídios à estrutura de Sm-14.

RESUMO DA INVENÇÃO

Esta invenção está relacionada com um antígeno que confere imunidade protetora contra infecção helmíntica em seres humanos e animais e o processo de vacinação para a imunoprofilaxia de doenças helmintológicas de interesse médico e veterinário.

O objeto da presente invenção é uma vacina contra a infecção causada por Fasciola hepatica em gado, bode, cabras e ovelhas.

Outro objeto da presente invenção é uma vacina contra infecção causada por Schistosoma mansoni e por todas as outras espécies de Schistosoma que são responsáveis por infecções e doenças em serem humanos e em animais.

Ainda outro objeto desta invenção é uma vacina contra infecção causada por todas as espécies de helmintos de interesse médico e veterinário.

Um objeto mais extenso da presente invenção é o uso da rSm-14 no diagnóstico de esquistosomose e Fasciolose.

O objetivo adicional é um método de desenvolvimento de uma vacina contra Schistosoma humano através do uso do mesmo antígeno vacinante na imunoprofilaxia de doenças causadas por espécies diferentes de parasitas que afetam serem humanos e vários animais.

Um objetivo ulterior é a molécula de Sm-14 que possui uma estrutura tridimensional, definida conforme a presente invenção.

BREVE DESCRIÇÃO DAS FIGURAS

A Figura 1 mostra um gel da preparação final do antígeno purificação de (rSm 14) comparado ao SE.

A Figura 2 mostra a estrutura tridimensional do rSm-14 prognosticado por modelagem computadorizada.

A Figura 3 mostra a avaliação do nível de proteção do rSm-14 de acordo com a experiência 1.

A Figura 4 mostra a avaliação do nível de proteção do rSm-14 de acordo com a experiência 2.

A Figura 5 mostra a avaliação do nível de proteção do rSm-14 de acordo com a experiência 3.

A Figura 6 mostra a avaliação do nível de proteção do rSm-14 de acordo com a experiência 4.

A Figura 7 mostra a soma dos resultados das experiências 1, 2, 3 e 4.

A Figura 8 mostra a vacinação em camundongos suíços com rSm-14 contra a infecção causada por Fasciola hepatica.

A Figura 9 mostra o fígado de um animal não vacinado que foi infectado por Fasciola hepatica.

A Figura 10 também mostra o fígado de um animal não vacinado que foi infectado pela Fasciola hepatica.

A Figura 11 mostra o fígado de um animal vacinado que foi infectado por Fasciola hepatica.

DESCRIÇÃO DETALHADA DA INVENÇÃO

O método de desenvolvimento de uma vacina contra as espécies de Schistosoma humano através do uso do mesmo antígeno vacinante na imunoprofilaxia de doenças causadas por diferentes espécies de parasitas que afetam seres humanos e vários animais pode ser descrita através dos seguintes

procedimentos:

- a obtenção do isolamento do antígeno comum de reação cruzada (que é o Sm-14 segundo a concretização preferida da presente invenção) o qual é altamente preventivo contra doenças de animais e seres humanos.

- esse antígeno testado como uma vacina para a imunoprofilaxia de doenças de animais em hospedeiros experimentais e definidos para o parasita que causa a infeção e/ou doença.

- análise de informação oriunda da vacinação do animal hospedeiro, isto é, ruminantes domésticos, enfocando todas as questões e pré-requisitos para o desenvolvimento final da vacina contra uma dada doença humana como a patologia e toxicologia.

Usando o método conforme a presente invenção, podemos alcançar um antígeno que seja simultaneamente bastante eficaz como vacina contra 2 tipos de doenças parasitárias, em seres humanos e animais domésticos. Conforme a concretização preferida da presente invenção, as doenças parasitárias de ambos, seres humanos e animais domésticos são, respectivamente Fasciolose e Esquistosomose bem como outras doenças helmínticas as quais afetam especificamente seres humanos e diferentes espécies de animais.

Um dos antígenos na mistura do complexo SE, o Sm-14 foi clonado e exibiu uma homologia significante com proteínas que ligam ácido graxo, e também com o antígeno Fasciola hepatica Fh 15. Esse antígeno de reação em cruzada, isto é Sm-14 em sua forma recombinante, confere imunidade protetora contra ambos, Esquistosomose e Fasciolose.

Nós demonstraremos aqui a habilidade da forma recombinada do Sm-14 para conferir uma alta proteção contra Fasciola hepatica, Schistosoma mansoni, bem como todas as espécies de Schistosoma e Echeinococcus e outros helmintos putativos que são patológicos aos seres humanos e aos animais. Os níveis de

proteção alcançados pela vacinação experimental em centenas de animais, têm demonstrado que o Sm-14 é a maior molécula protetora derivada do SE e que é o candidato para ambas as vacinas anti-Schistosoma e anti-Fasciola.

A presente invenção irá descrever nos termos dos exemplos mas não estará limitada a eles.

EXEMPLO 1

O processo para a obtenção, caracterização e purificação do Sm-14 recombinado será demonstrado como se segue:

Fase 1:

A transição de um extrato salino protetor (SE) para a vacina molecular foi obtida como se segue:

a) O arquivo de uma cepa gt 11 cDNA LE (preparado através de vermes adultos de cepas endêmicas do LE de Schistosoma mansoni) foi protegida com soro imunológico anti-SE derivado de indivíduos plenamente protegidos (isto é, coelhos e coelhos com soro de "Alta Proteção" conforme anteriormente descrito neste documento).

b) Uma espécie de clone cDNA que foi reconhecido por coelhos com soro de alta proteção anti-SE foram os escolhidos dentre outras espécies, por ofereceram sinais altamente intensos.

c) A sequência e caracterização revelaram a proteína de 14 KDa chamada Sm-14 (o nucleotídio e a sequência deduzida de aminoácido já faz parte do trabalho publicado por Moser, Tendler et al.).

Um exemplo prático de como se conduzir a produção do clone cDNA encontra-se descrito no estado da arte.

Fase 2:

A expressão de Sm-14 em um sistema vetor eficiente.

O método para conduzir isso até o PDS-14 está descrito no estado da arte

(A 14-KDa Schistosoma mansoni Polypeptide Is Homologous to a gene Family of Fatty Acid Binding Proteins, The Journal of Biological Chemistry, Vol 266, No. 13, publicado em 5 de Maio, pp. 8447-8454, 1991) bem como a identificação e os resultados da sequência de cDNA clonados e foi incorporado a título de referência.

O anti-soro produzido em coelhos imunizados com o extrato de Schistosome foi utilizado para proteger o cDNA S. mansoni adulto de arquivo (anteriormente descrito). Um clone designado Sm-14 foi purificado em placa após três sucessões de imuno-proteção. O fago recombinante foi lisogenizado em E. coli Y1089 e induzido para expressar uma proteina de fusão de Sm-14 - beta galactosidase de 122 KDa. A proteína foi purificada por eletroforese de gel de poliacrilamida - SDS, e os anti-corpos para a proteína de fusão foram acrescentados em um coelho.

A sub-clonagem de Sm-14 e sua expressão no vetor atual no qual foram feitos os ensaios de vacinação contra Schistosoma e Fasciola é descrita abaixo:

Eliminar a estrutura de leitura aberta para o Sm 14 a partir da construção original pDS - Sm 14 por clivagem com Bam HI e Hind III.

O fragmento obtido foi ligado em pGEMEX-1 (Promega) clivado com as mesmas enzimas.

Fase 3:

A construção resultante que por sua vez resultou no gene que está na estrutura para expressão como uma proteina de fusão com a proteína do gene 10 T7, sob o controle de um promotor de polimerase de RNA T7, foi usado para transformar a cepa de E. coli BL 21 (DE 3) que contém o gene de polimerase de RNA T7 sob controle de lac UV. A cepa de E. coli BL 21 (DE 3) foi usada para expressão de proteína recombinante. Outras cepas de E. coli podem ser alternativamente empregadas para a mesma finalidade, bem como outros sistemas de expressão, por exemplo PDS - 14 conforme o estado da técnica.

Fase 4:

As colônias contendo o plasmidio recombinante cresceram durante a noite, e a expressão de polimerase de RNA T7 induzida pela adição de IPTG durante subsequente crescimento da fase logarítmica.

Esse procedimento resultou na expressão de uma proteína de fusão com um peso molecular estipulado em 40 KDa (14 KDa a partir de Sm 14 e 26 KDa a partir da proteína do gene 10).

Fase 5:

As células da bactéria foram coletadas por centrifugação (5000 rpm/10 min) e re-suspensas em tampão de lise (50 mM Tris/HCl; pH 7,5; 2 mM EDTA; 1 mM DTT; 2 mg/ml lisozuma) e incubadas em gelo por 15 minutos. Os lisados foram então sonificados por 2 ciclos de 30 segundos e re-centrifugados.

A pelota foi re-suspensa em um tampão de lavagem (50 mM Tris - HCl; pH 7,5; 10 mM EDTA; 1 mM DTT; 0,5% Triton x-100) e centrifugada.

Fase 6:

Seguindo uma rodada adicional de ressuspenação e centrifugação, a pelota final foi re-suspensa em água. Foi então feito SDS-PAGE, o antígeno purificado por eletroeluição e estocado em temperaturas variando de -70 ^{o}C a -200 ^{o}C até o uso.

A Figura 1 mostra o grau de pureza do rSm 14 e a alta eficiência da expressão.

A análise de eletroforese de gel de poliacrilamida dos antígenos de totais do SE S. mansoni e o Sm 14 purificado foi transferido para papel de nitrocelulose. As colunas 1 - 3, SE e Sm-14 re-dissolvidos em 10 e 15% de SDS - PAGE respectivamente, e coloridos com C. Azul. As colunas 2 e 4 imunoblot. A coluna 2 foi experimentada com anti-soro policlonal a partir de coelhos imunizados com SE. A coluna 4 com anti-soro de proteína de fusão de anti-Sm 14 de coelho.

Os marcadores de baixo padrão molecular são mostrados em ambos os lados da figura.

Em seguida será feito referência à Figura 2 que mostra a estrutura tri-dimensional do Sm 14.

A modelagem por computador da estrutura do Sm 14 de acordo com a presente invenção é suportada na alta homologia conhecida do Sm 14 com proteínas para as quais a estrutura cristalina já foi determinada. Isto permite uma estrutura tri-dimensional detalhada e confiável do Sm 14.

A estrutura tri-dimensional ensina que (1) o Sm 14 possui uma proteína no formato de um barril; (2) o ácido graxo se liga dentro do barril; (3) o barril é formado por 10 lâminas beta dobradas; (4) as lâminas são ligadas por curtas voltas; (5) as voltas exibem divergência entre membros da família de ácidos graxos ligando proteínas e é responsável pela anti-genicidade do Sm 14.

EXEMPLO 2

O Exemplo 2 inclui os experimentos 1 a 4. Os protocolos dos experimentos 1 a 4 foram realizados como descrito abaixo, e mostram a atividade protetora de SE e Sm 14 em camundongos suíços.

Os protocolos de imunização com SE (300 ig/ml/por dose/animal) e proteína de fusão de Sm 14 (10 ug/ml/dose) foram realizados com o protocolo de imunização que consiste de duas doses do antígeno, com ou sem adjuvante de Freund's, dadas aos camundongos naturais em intervalos de 7 dias por injeção subcutânea seguindo uma dose intensificadora 21 dias após a segunda dose. Os intervalos entre a aplicação das doses vacinantes podem ser variados. Após um intervalo de 60 dias (que também pode ser variado, por exemplo 45 dias) os animais foram desafiados com 100 cercariae.

A proteção total para cada grupo de animais (imunizados/desafiados e

respectivos controles) foi calculada como segue.

$$C - V/C \times 100$$

onde C = parasitas recuperados dos controles; e V = parasitas recuperados dos animais vacinados.

Os resultados são mostrados na Tabela I

Tabela I

ATIVIDADE PROTETORA DE SE E Sm 14 EM CAMUNDONGOS SW

Imunização Antigeno (3 doses)	nº camundongos	Carga Parasitaria	Proteção %
Exp 1.: 10 ug Sm 14 + FCA	20	12.1	50.6
10 ug Sm 14	19	9.9	59.6
10 ug Gene 10 + FCA	22	28.4	0
10 ug Gene 10	22	27.7	0
PBS	12	24.5	0
Exp 2.: 300 ug SE + FCA	21	7.8	72.1
300 ug SE	20	12.9	53.9
10 ug Sm 14 + FCA	10	9.6	65.7
10 ug Sm 14	14	13.6	51.4
PBS	8	28.0	0
Exp 3.: 300 ug SE + FCA	11	11.6	56.7
300 ug FCA	10	25.9	0
10 ug Sm 14 + FCA	11	10.1	62.3
10 ug Sm 14	12	8.6	67.9
PBS	8	26.8	0
Exp 4.: 300 ug FCA	10	23.2	0
10 ug Sm 14 + FCA	9	10.1	64.0
10 ug Sm 14	9	12.5	55.3
PBS	7	28.0	0

Diferentes grupos de controle caracterizados por sexo e idade correspondentes ao camundongos suiços, simultaneamente desafiados com o mesmo número e conjunto cercariae de S. mansoni, foram usados como controles da infecção para cada experimento individual. Esses animais receberam somente injeções paralelas de PBS (Fosfato Neutro Salino). Grupos adicionais de controle para a proteína de fusão (gene 10) e o adjuvante (o adjuvante completo de Freund) foram também incluídos.

No experimento 1, a atividade protetora do rSm-14 com ou sem adjuvante (FCA) foi analisada paralelamente à atividade da proteína do gene 10, como pode ser observado na Tabela II. As cargas médias dos vermes recuperados dos camundongos vacinados com a proteína purificada ao gene 10, com ou sem FCA, foram virtualmente as mesmas das cargas parasitárias colhidas dos animais do grupo de controle PBS.

Nos experimento 2, a atividade protetora induzida pelo rSm-14 e rSm-14 com FCA foi medida em comparação com a vacinação com SE (com ou sem FCA).

Os experimentos 3 e 4 foram elaborados para testar a atividade do FCA sozinho e a reprodutibilidade da atividade protetora induzida pela vacinação com rSm-14.

Em todos os experimentos foi conclusivamente demonstrada a alta capacidade do rSm-14 para induzir níveis significativamente elevados de imuno proteção contra novas infecções desafiadoras de camundongos com S. mansoni.

A análise estatística dos dados apresentados mostra que a carga parasitária recuperada dos grupos vacinados é significativamente mais baixa ($p < 0,05$) do que a média do número de parasitas abrigados em animais infectados não-vacinados.

EXEMPLO 3

Este exemplo mostra uma atividade protetora do SE e rSm-14 em coelhos.

Os protocolos de imunização são os mesmos que aqueles usados nos camundongos suíços do Exemplo 2.

A Tabela II mostra as quantidades de dose por animal. Os coelhos foram desafiados com 1000 cercariae (ao invés de 100 como no Exemplo 2).

A Tabela II mostra a capacidade do rSm-14 de induzir níveis significativamente altos de imuno proteção contra infecção desafiante em coelhos com S. mansoni.

Além disso, este exemplo torna clara a atividade do rSm-14 com um antígeno isolado em comparação com a mistura SE.

Os resultados são mostrados na Tabela II.

Tabela II

ATIVIDADE PROTETORA DE SE E rSm 14 EM COELHOS (N. ZELANDIA)

Imunização Antigeno + FCA 3 doses	Number de coelhos	X =sem	Proteção (%)
600 ug SE + FCA	4	7.4 ± 3.9	93
80 ug Sm 14 + FCA	4	12.0 ± 4.1	89
Controle	4	109.5 ± 11.0	-

EXEMPLO 4

Este exemplo demonstra os experimentos 1 a 4 do Exemplo 2 (o que significa que os mesmos protocolos de imunização foram utilizados) mas com uma diferente metodologia para avaliar a proteção.

Esta metodologia é baseada no estabelecimento de resistência induzida por vacina, por meio de uma análise populacional das frequências das cargas parasitárias através da distribuição das cargas parasitárias numa série de faixas de parasitas.

Os resultados são mostrados na Tabela III.

Tabela III

Atividade Protetora de rSm 14 em camundongos outbred

Distribuição de Frequência da Carga Parasitária

Experimento 1

Carga parasit.	Sm14 + FCA	Sm 14	Gene 10 + FCA	Gene 10	Controle (PBS)
0 - 10	65, 0	57, 9	-	-	-
11 - 20	20, 0	31, 6	4, 5	-	19, 7
21 - 30	15, 0	10, 5	59, 1	68, 2	61, 7
31 - 40	-	-	31, 8	27, 3	10, 5
41	-	-	4, 5	4, 5	8, 1
N = camu./gp.	20	19	22	22	88

Experimento 2

Carga Parasit.	SE + FCA	SE	Sm14 + FCA	Sm14	Controle (PBS)
0 - 10	76, 2	40, 0	60, 0	35, 7	-
11 - 20	23, 8	55, 0	40, 0	64, 3	19, 7
21 - 30	-	5, 0	-	-	61, 7
31 - 40	-	-	-	-	10, 5
41	-	-	-	-	8, 1
N = camun./gp	21	20	10	14	88

Experimento 3

Carga Parasit.	SE + FCA	FCA	Sm14 + FCA	Sm14	Controle (PBS)
0 - 10	77, 8	-	63, 6	66, 7	-
11 - 20	11, 1	40, 0	36, 4	33, 3	19, 7
21 - 30	11, 1	30, 0	-	-	61, 7
31 - 40	-	30, 0	-	-	10, 5
41	-	-	-	-	8, 1
N = camun./gp	9	10	11	12	88

Experimento 4

Carga Parasit.	FCA	Sm 14 + FCA	Sm14	Controle (PBS)
0 - 10	-	44, 4	22, 2	-
11 - 20	40, 0	44, 4	77, 8	19, 7
21 - 30	30, 0	11, 2	-	61, 7
31 - 40	30, 0	-	-	10, 5
41	-	-	-	8, 1
N = camun./gp	10	9	9	88

De acordo com a Tabela III, o recombinante purificado Sm-14 estimula um nível de proteção que não foi significativamente diferente daquele do SE intacto, se julgados pelos níveis médicos da carga parasitária (Tabela I).

Os níveis de proteção atingidos com o SE são consistentes com os resultados previamente publicados. É de especial interesse o fato de que um nível semelhante de proteção é atingido com ou sem adjuvante, o que é um bom sinal para o uso do antígeno em seres humanos. Além disso, o fato de que tivemos sucesso com o antígeno na proteção de camundongos suíços, nascidos de intercruzamento de linhagem não relacionadas, mostra que restrições genéticas do sistema imunológico não resultam em sérias variações da resposta protetora.

Como pode ser visto na Tabela III, foram observadas formas completamente diferentes na distribuição da carga parasitária entre os grupos vacinado e não-vacinado.

Particularmente digna de atenção é a diferença no número de camundongos no grupo com 0-10 parasitas. Em seguida a uma infecção de desafio ao cercariae/ camundongo, nenhum dos animais não-vacinados tinha níveis de infecção naquela faixa e o pico de frequência (60%) para os animais infectados (não vacinados) estava na faixa de 21-30 parasitas. Em contraste, o pico de frequência (64,5%) para os camundongos vacinados, quer com SE ou com Sm-14, caiu na faixa de 0-10 parasitas/camundongo.

Como pode ser visto, de acordo com a presente invenção é de particular interesse que essencialmente o efeito protetor do complexo da mistura SE pode ser integralmente reproduzido com este único antígeno.

Experiências com outros antígenos definidos derivados de SE (glutachiona-S-transferase e paramiosina) não resultaram no mesmo alto nível de proteção. Como mencionado acima, o Sm-14 também tem um significativo nível de homologia com vários ácidos gordurosos que ligam proteínas.

Os resultados dos experimentos 1 a 4 da Tabel III são demonstrados graficamente nas Figuras 3 a 6.

As Figuras 3 a 6 correspondem aos experimentos 1 a 4. Nessas figuras é possível avaliar a proteção pela análise dos perfis da carga parasitária dos grupos vacinados e não-vacinados.

A Figura 7 mostra os resultados em conjunto.

EXEMPLO 5

Neste exemplo, camundongos vacinados foram desafiados com 500 e 1000 cercariae por animal ou desafiados 2 ou 3 vezes (infecção de 100 cercariae por animal) com uma semana de intervalo entre cada vez. Como se notará, os tamanhos e os números das infecções desafio são variados.

A proteção induzida por 3 doses de 10 ug de proteína (rSm-14) injetada permanece acima de 50% contra uma única infecção desafio de 500 ou 1000 cercariae/animal.

O mesmo efeito é observado quando a infecção desafio corre 100 cercariae/animal é repetida 2 ou 3 vezes, com intervalo de uma semana entre cada uma.

Os protocolos para este Exemplo serão incorporados aqui.

Os dados do Exemplo 5 estão sintetizados nas Tabelas IV e V, respectivamente.

Protocolo - Vacinação

rSm 14 + FCA

40 camundongos
10 ug (x3) 7/7 dias

Infeção
45 dias
rota subcutânea

Grupo 1

20 camundongos
1.000 cercariae/
camundongo

Grupo 2

20 camundongos
500 cercariae/
camundongo

Perfusão
45 dias

Avaliação de
Carga Parasitária
Grupos 1-2

Protocolo - Vacinação

rSm 14 + FCA

Grupo 1	Grupo 2	Grupo 3
Infecção I	**Infecção I**	**Infecção I**
20 camundongos 100 cercariae	20 camundongos 100 cercariae	20 camundongos 100 cercarie
	7 dias	7dias
	Infecção II	**Infecção II**
	20 camundongos 100 cercariae	20 camundongos 100 cercariae
		7 dias
		Infecçãoiii 20 camundongos 100 cercariae

TABELA IV

ATIVIDADE PROTETORA DE rSm 14 EM CAMUNDONGOS COMO UMA FUNÇÃO DA VARIAÇÃO DE INFECÇÃO DESAFIO

VACINAÇÃO COM rSm 14 + FCA CONTRA DIFERENTES INFECÇÕES

GRUPOS	CAMUNDONGOS	NÚMERO DE CERCÁRIAS/CAMUNDONGO	X	PROTEÇÃO (%)
1	20	1.000	58 ± 13.2	65.9
CONTROLE	20	1.000	170 ± 15.0	-
2	20	500	31.5 ± 2.3	49.7
CONTROLE	20	500	62.6 ± 2.1	-

TABELA V

ATIVIDADE PROTETORA DE rSm 14 EM CAMUNDONGOS COMO UMA FUNÇÃO DE INFECÇÕES DESAFIO MULTIPLAS

VACINAÇÃO COM rSm 14 + FCA CONTRA DIFERENTES INFECÇÕES

GRUPOS	CAMUNDONGOS	NÚMERO DE CERCARIAE/CAMUNDONGO	X	PROTEÇÃO (%)
1	20	100	11.2 ± 1.09	58.8
CONTROLE	20	100	27.2 ± 2.2	-
2	20	100 (X2)	33.0 ± 1.7	57.3
CONTROLE	20	100	52.6 ± 1.5	-
3	20	100 (X3)	42.3 ± 2.3	59.2
CONTROLE	20	100	47.3 ± 3.3	-

EXEMPLO 6

Para demonstrar a atividade dos soros dos pacientes de esquistosomose contra a proteína que liga os ácidos graxos do Schistosoma mansoni - rSm-14, o exemplo é executado como segue.

Os soros dos pacientes humanos de uma área endêmica no Brasil e os soros de jovens vivendo fora da área endêmica são examinados por imunobloting contra o antígeno recombinante Sm-14. Os pacientes são classificados em grupos de acordo com a forma clínica e os ovos são contados. O diagnóstico parasitológico é obtido pelo método Kato-Katz.

Os resultados mostram que o soro de todos os indivíduos infectados reconheceram rSm-14 no imunobloting, independentemente da idade, carga parasitária ou forma clínica, refletindo assim a imunogenicidade do rSm-14.

EXEMPLO 7

Este exemplo mostra a vacinação de camundongos suiços com rSm-14 contra a infecção de Fasciola hepatica e a completa proteção atingida contra a Fasciolose.

O Exemplo 7 foi executado como segue.

Dois grupos de 15 camundongos foram imunizados com rSm-14 com ou sem adjuvante. O protocolo da vacinação é: (a) duas injeções semanais do antígeno (dose de 10 ug de rSM-14 por animal) emulsificada ou não em FCA (adjuvante); (b) aplicado uma nova dose de injeção de antígeno três semanas depois e (c) quarenta e cinco dias após a terceira dose, eles foram desafiados com três metacercariae de Fasciola hepatica e sacrificados trinta dias após a infecção.

Este exemplo mostra os antígenos protetores de reação cruzada entre diferentes helmintos como Schistosomas e Fasciola hepatica.

Foi recentemente anunciado que um antígeno denominado FSh15 clonado a partir do parasita relacionado, Fasciola hepatica, tem um significativo nível de

homologia ao nível previsto da sequência de animo ácido com Sm-14 e apresenta resultados mostrando ser o Sm-14 o homólogo desta proteína na Fasciola hepatica.

O recombinante Sm-14 foi assim testado como um antígeno de vacinação contra a infecção de Fasciola hepatica conforme descrito neste Exemplo.

Segnem-se referências às figuras 9, 10 e 11, mostrando o fígado de animais não vacinados (Figs. 9 e 10) versus animais vacinados (Fig. 11).

Depois do exame parasitológico para avaliar a infecção pela Fasciola hepatica com animais vacinados com o rSm-14, e não-vacinados (controles), inoculadas com a mesma infecção por três metacercarie por animal, o fígado, os intestinos e outros órgãos foram examinados pelos processos histológicos clássicos para se avaliar a patologia desenvolvida nos animais dos dois grupos. Deve ser salientado que principalmente o fígado e os intestinos são os órgãos mais afetados pela Fasciola hepatica e, portanto, foram extensivamente examinados.

Assim, trinta dias depois da infecção oral pelo método clásnico com três metacercariae de Fasciola hepatica por camundongo, os animais foram sacrificados para uma avaliação da carga infecciosa adquirida na presença da prévia vacinação com rSm-14 em comparação com os animais não vacinados. Os órgãos foram fixados em uma solução Milloning, cortados, coloridos pela técnica de Hematoxilina-Eosina, e examinados sob o microscópio ótico.

Está conclusivamente demonstrado pelas Figuras 8, 9, 10 e 11 que o rSm-14 é capaz de induzir a proteção contra a infecção de Fasciola hepatica, com base nos dados parasitológicos e patológicos. Dos animais vacinados com rSm-14, virtualmente nenhum indivíduo adquiriu a infecção após exposição de três metacerariae de Fasciola hepatica (dose máxima permitida para camundongos). Ao contrário, todos os animais de controle não-vacinados foram infectados depois de igualmente expostos.

Do ponto de vista patológico, o parenquima do fígado de todos os

indivíduos que foram vacinados com rSm-14 não mostram nenhuma alteração relacionada com a infecção da Fasciola hepatica; com a exceção de pequenas áreas fibróticas ao nível da cápsula de Glisson. Essa descoberta mostra que os parasitas desafiantes foram mortos por efeito da vacinação, muito cedo no seu ciclo de vida no hospedeiro vertebrado. Ao contrário, todos os animais não vacinados/infectados exibiram extensas áreas de destruição de hepatócitos com regiões de severa hemorragia que se estendem até a cápsula de Glisson.

Como pode ser visto nas Figuras 9 e 10, verificou-se extensa destruição do parenquima, juntamente com a presença de parasitas adultos em vários indivíduos.

REIVINDICAÇÕES

1- Sm 14 como um antígeno caracterizado por possuir um peso molecular de 14 a 15 KDa derivado de Schistosoma mansoni onde a sequência de amino ácido está estabelecida em Ã 14 KDa Schistosoma mansoni Polypeptide Is Homologous to a gene Family of Fatty Acid Binding Proteins" e o polipeptideo tem a capacidade de ligar ácidos graxos, o dito Sm 14 sendo formado por uma cadeia polipeptídea dobrada em dez lâminas de beta dobradas, ligadas por curtas voltas, conforme mostrado na Figura 2.

2- Sm 14 caracterizado por ser obtido conforme o seguinte vetor de expressão:

fusão do cDNA do Sm 14 para a porção do bacteriófago T7 do gene 10 que codifica os 260 primeiros amino ácidos da proteína do capsídeo T7 na forma de plasmídeo;

expressar a proteina de fusão por transformação de uma cepa apropriada de E. coli;

purificação inicial da proteina de fusão expressada por sonificação, repetir o lise e centrifugar;

purificação final por eletroeluição a partir de géis de SDS-PAGE.

3- Sm 14 de acordo com a reivindicação 2 caracterizado pela expressão ser alcançada usando a cepa de E. coli BL 21 (DE 3).

4- Sm 14 de acordo com a reivindicação 2 caracterizado pelo dito Sm 14 poder ser obtido por síntese química de toda ou parte da cadeia de polipeptideo.

5- Uso do antígeno Sm 14 caracterizado por ser como uma vacina contra as doenças causadas pelo Schistosoma mansoni.

6- Uso do antígeno Sm 14 caracterizado por ser como uma vacina contra as doenças causadas pela Fasciola hepática.

7- Uso do antígeno Sm 14 caracterizado por ser como uma vacina contra as doenças causadas por todas as espécies de Schistosoma em humanos e animais.

8- Uso do antígeno de Sm 14 caracterizado por ser como uma vacina contra doenças causadas por helmintos.

9- Uso de acordo com as reivindicações 5 a 8 caracterizado por o Sm 14 estar em sua forma recombinante.

10- Uso do Sm 14 caracterizado pelo dito uso ser como diagnóstico de esquistosomose e fasciolose.

11- Método de aplicar uma vacina veterinária em humanos caracterizado por ter as seguintes etapas:

efetuar a isolação de um antígeno comum de reação cruzada que é protetor contra ambas as doenças de animais e humanos;

testar o antígeno como uma vacina para a imunoprofilaxia de doenças de animais no animal hospedeiro para o parasita que causa a infecção e/ou a doença.

analisar a informação derivada da vacinação do animal hospedeiro; e,

enfocar todas as questões relacionadas e pré-requisitos para a aplicação da vacina veterinária contra a doença em humanos

Fig 1

GEL DA PREPARAÇÃO FINAL DO ANTÍGENO
(purificação de rSm 14) EM COMPARAÇÃO AO SE

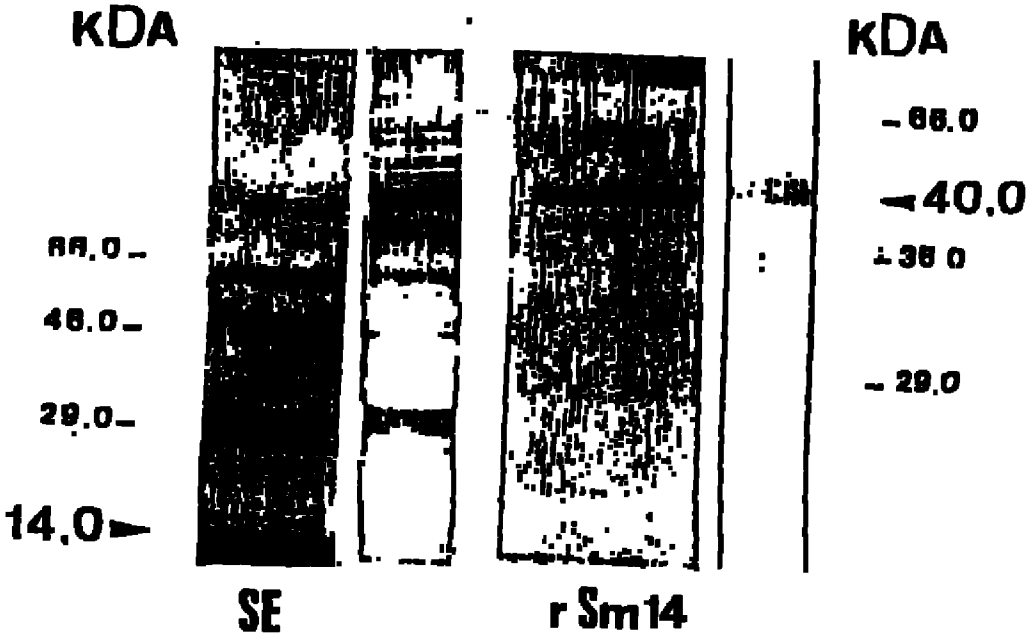


Fig 2

Fig 3

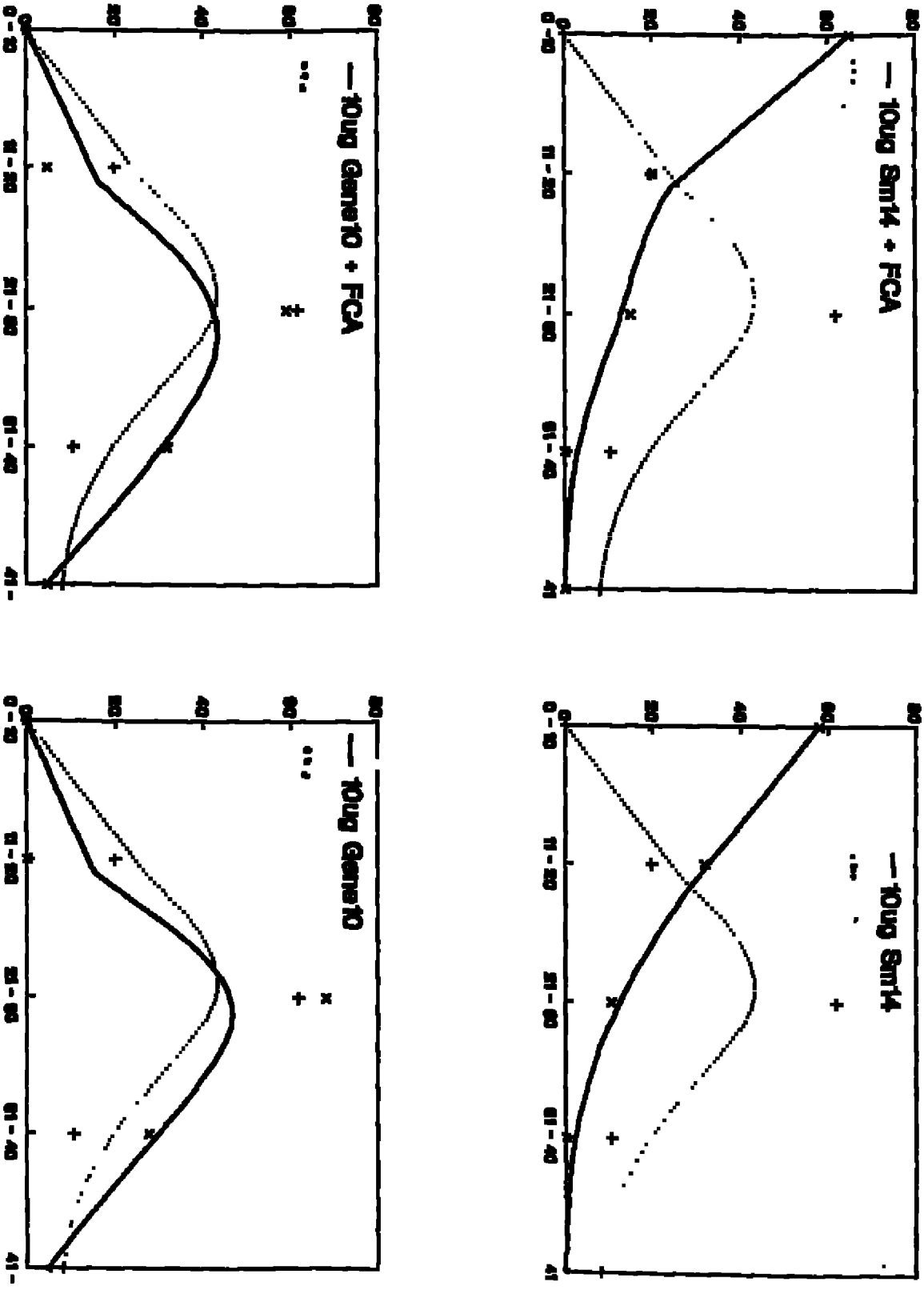


Fig 4

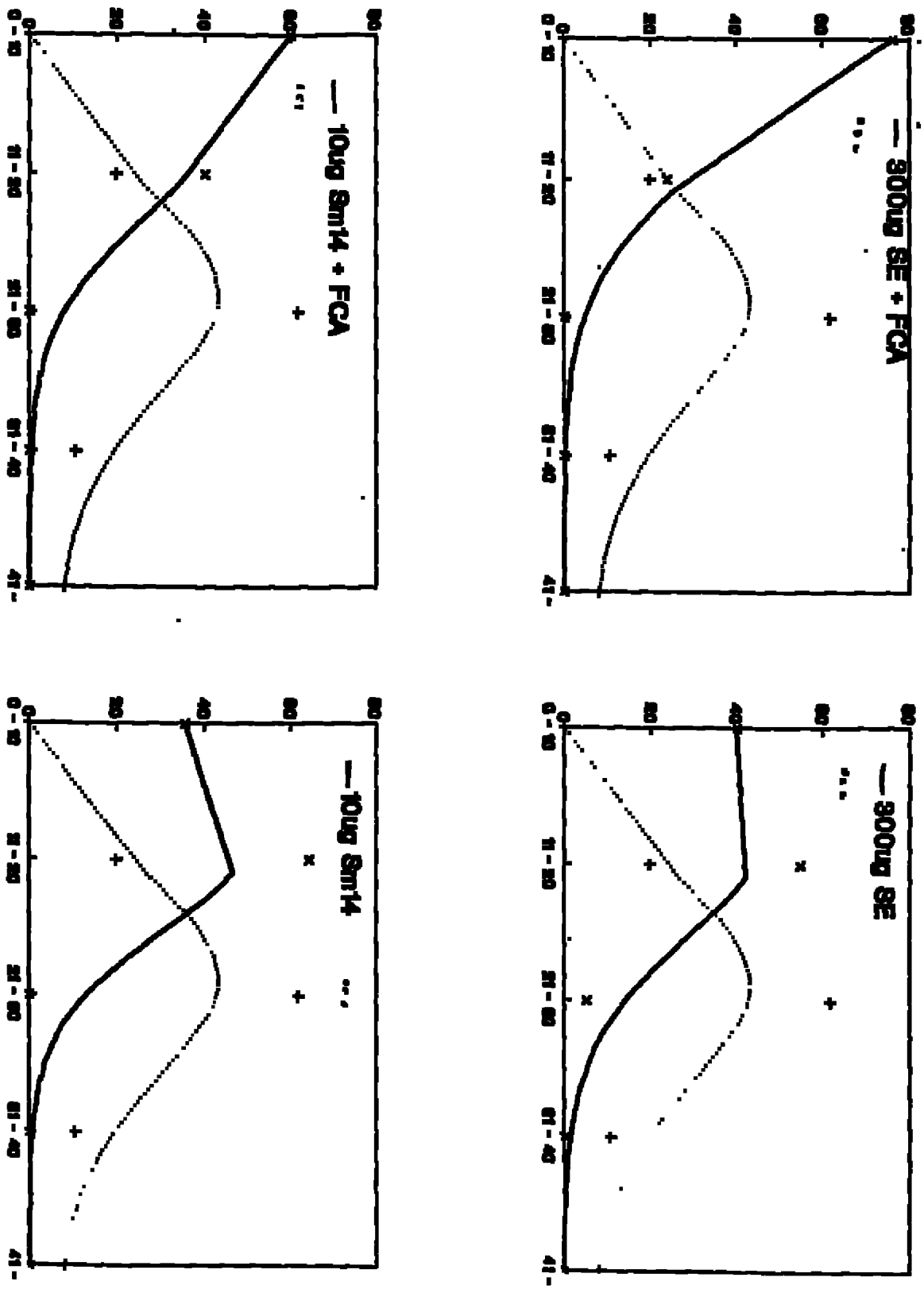
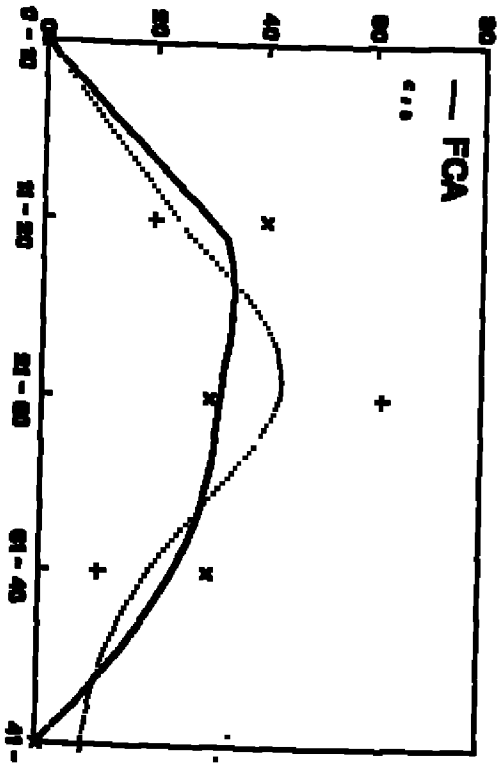
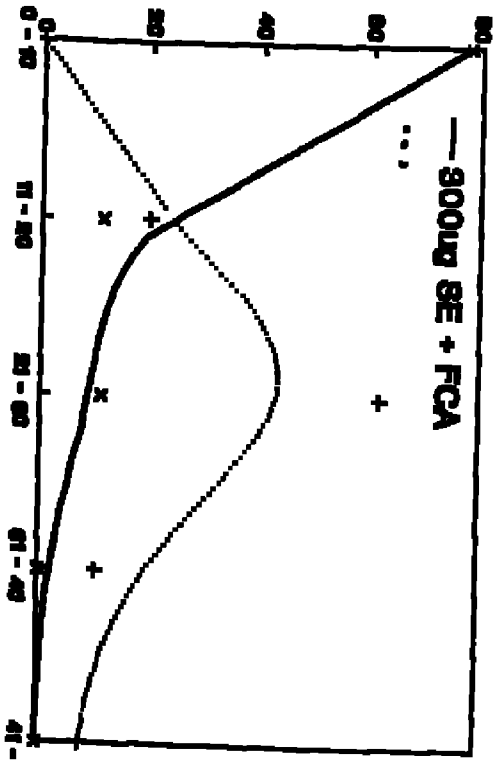
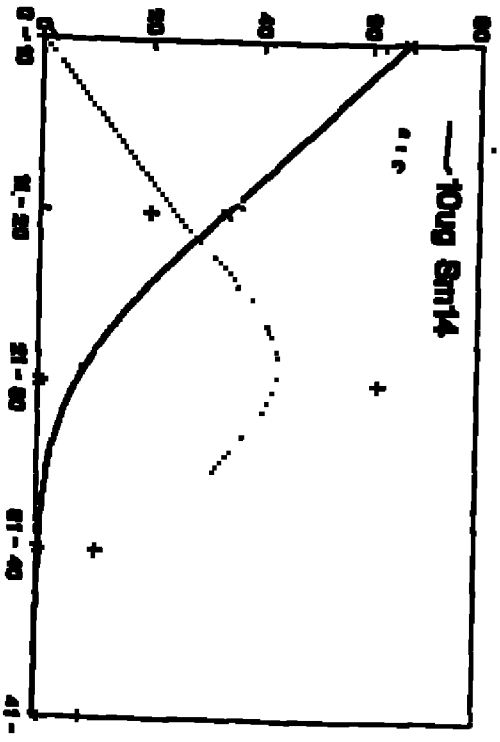


Fig 5







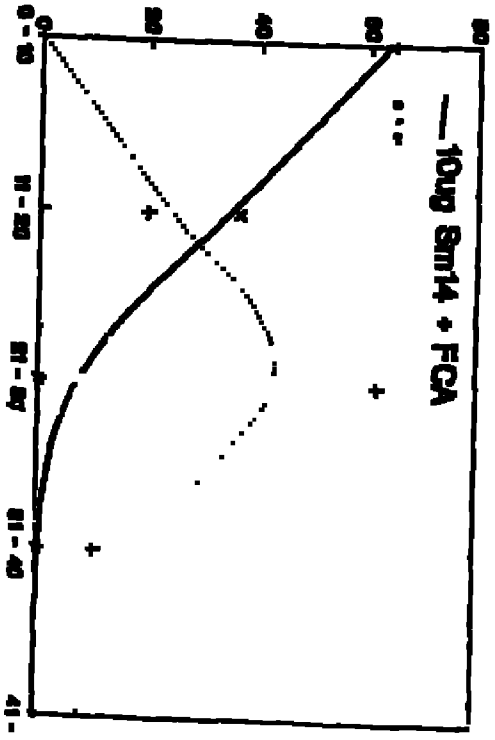
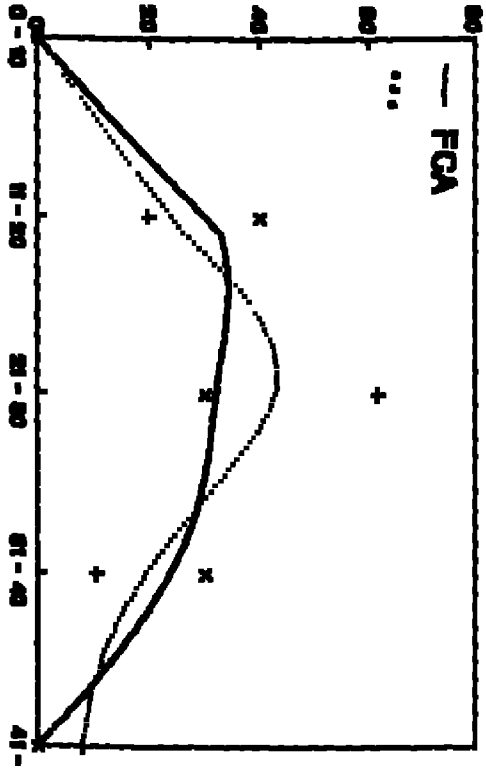
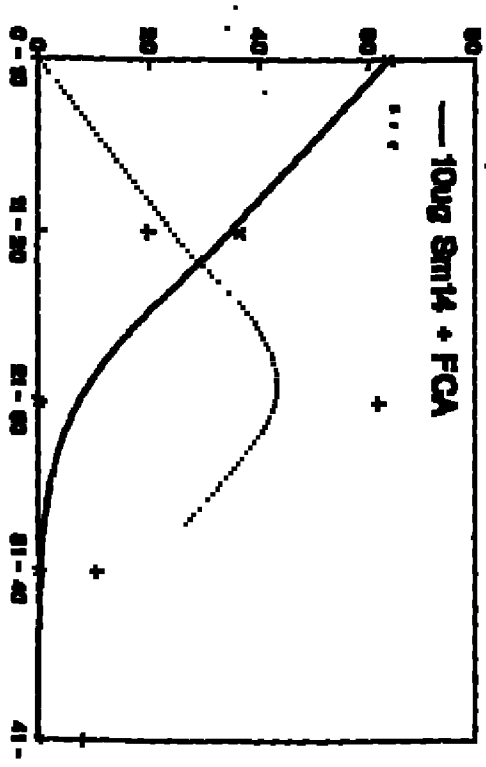
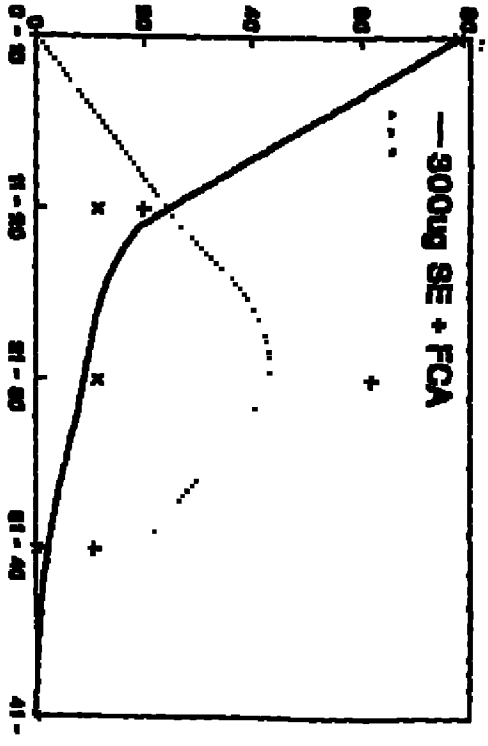


Fig 6

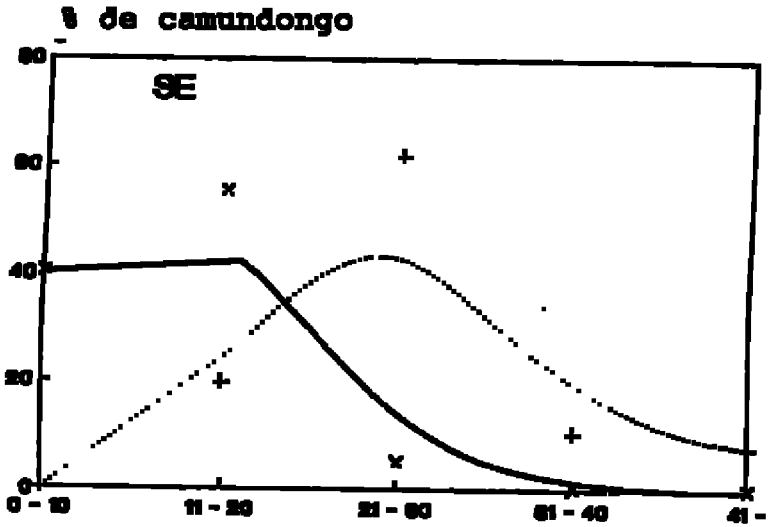

— FCA
0-10
11-20
21-30
31-40
41-


—10ug Sm14 + FCA
0-10
11-20
21-30
31-40
41-


—300ug SE + FCA
0-10
11-20
21-30
31-40
41-

SOMA DE RESULTADOS

Fig 7



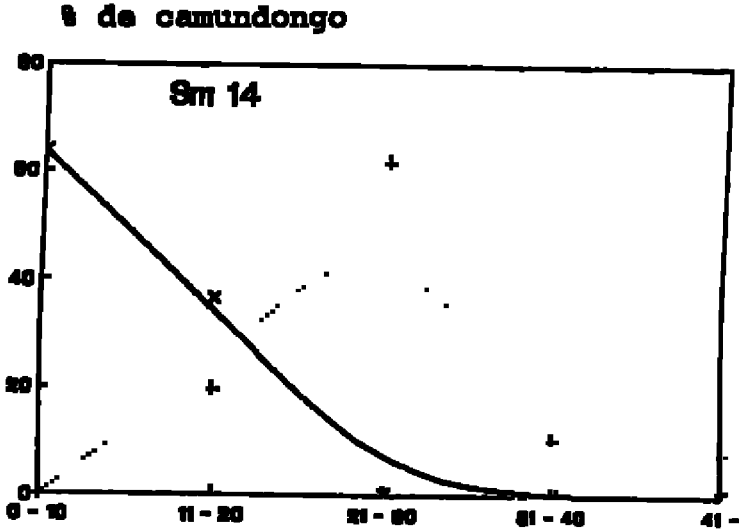
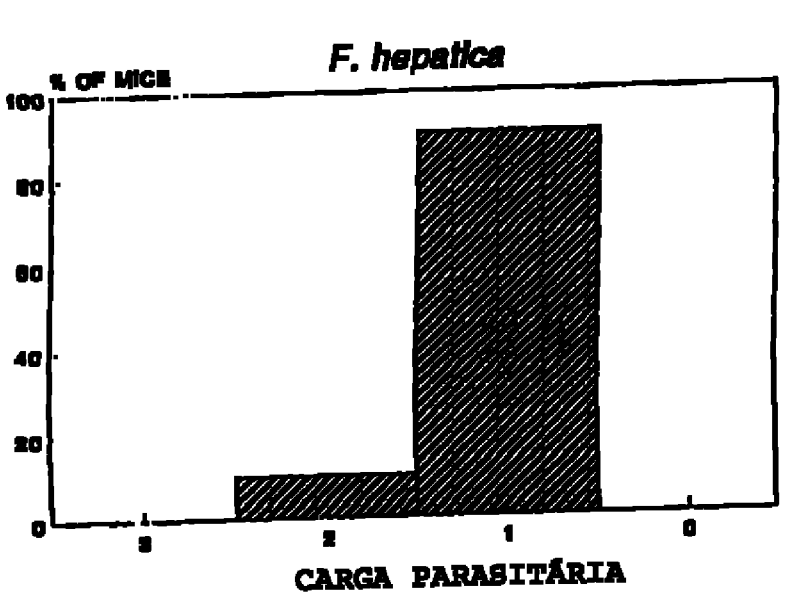


Fig 8

VACINAÇÃO DE CAMUNDONGOS SUÍÇOS COM rSm 14 CONTRA INFECÇÃO COM FASCÍOLA HEPÁTICA



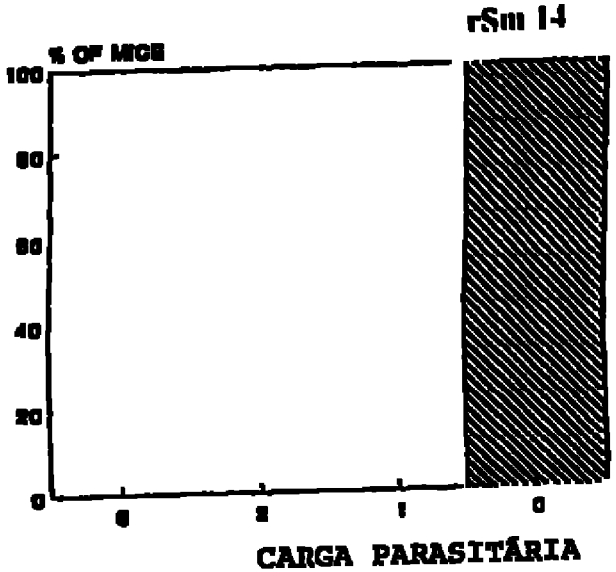
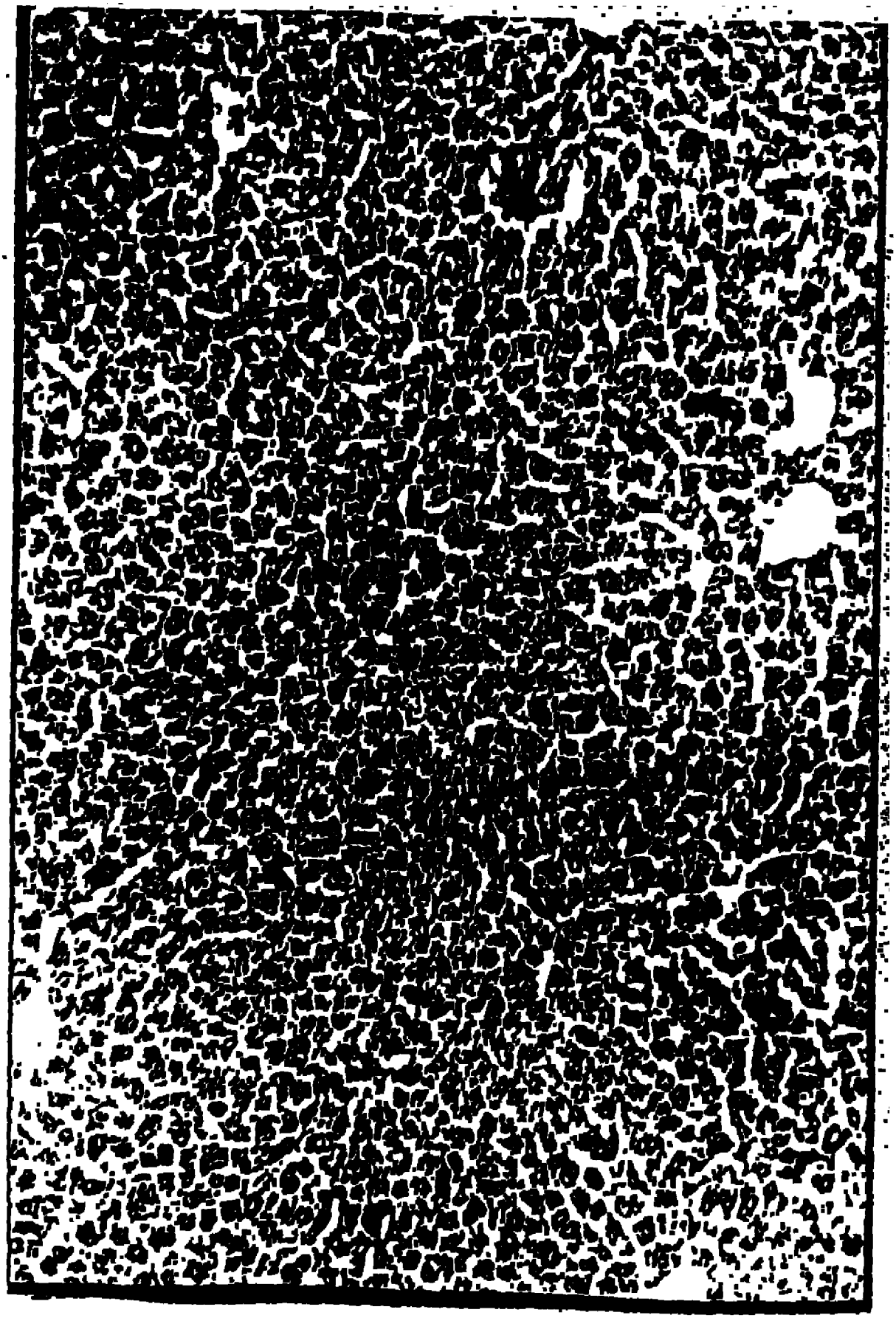


Fig. 9

Fig. 10

Fig. 11.



9305075

RESUMO

Patente de Invenção: "ANTÍGENO PARA CONFERIR IMUNIDADE PROTETORA CONTRA INFECÇÕES HELMÍNTICAS EM HUMANOS E ANIMAIS, E, PROCESSO DE VACINAÇÃO PARA APLICAÇÃO NA IMUNOPROFILAXIA DE DOENÇAS HELMINTOLÓGICAS DE INTERESSE VETERINÁRIO E MÉDICO"

Essa invenção se refere de forma geral a material antígeno derivado de helmintos capaz de induzir efetiva e duradoura proteção contra parasitas, em particular a antígenos que servem para intermediar imunidade protetora contra helmintos.

PLOS | NEGLECTED TROPICAL DISEASES

*Sm*10.3, a Member of the Micro-Exon Gene 4 (MEG-4) Family, Induces Erythrocyte Agglutination *In Vitro* and Partially Protects Vaccinated Mice against *Schistosoma mansoni* Infection

Vicente P. Martins[1,2,3,4], Suellen B. Morais[1,2], Carina S. Pinheiro[1,2,5], Natan R. G. Assis[1,2], Barbara C. P. Figueiredo[1,2], Natasha D. Ricci[1,2], Juliana Alves-Silva[1], Marcelo V. Caliari[6], Sergio C. Oliveira[1,2]*

1 Departamento de Bioquímica e Imunologia do Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **2** Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais (INCT-DT), CNPq MCT, Salvador, Bahia, Brazil, **3** Departamento de Biologia Geral do Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **4** Departamento de Biologia Celular do Instituto de Ciências Biológicas, Universidade de Brasília, Brasília, Distrito Federal, Brazil, **5** Departamento de Biointeração do Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Bahia, Brazil, **6** Departamento de Patologia Geral do Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Abstract

Background: The parasitic flatworm *Schistosoma mansoni* is a blood fluke that causes schistosomiasis. Current schistosomiasis control strategies are mainly based on chemotherapy, but many researchers believe that the best long-term strategy to control disease is a combination of drug treatment and immunization with an anti-schistosome vaccine. Numerous antigens that are expressed at the interface between the parasite and the mammalian host have been assessed. Among the most promising molecules are the proteins present in the tegument and digestive tract of the parasite.

Methodology/Principal Findings: In this study, we evaluated the potential of *Sm*10.3, a member of the micro-exon gene 4 (MEG-4) family, for use as part of a recombinant vaccine. We confirmed by real-time PCR that *Sm10.3* was expressed at all stages of the parasite life cycle. The localization of *Sm*10.3 on the surface and lumen of the esophageal and intestinal tract in adult worms and lung-stage schistosomula was confirmed by confocal microscopy. We also show preliminary evidence that r*Sm*10.3 induces erythrocyte agglutination *in vitro*. Immunization of mice with r*Sm*10.3 induced a mixed Th1/Th2-type response, as IFN-γ, TNF-α, and low levels of IL-5 were detected in the supernatant of cultured splenocytes. The protective effect conferred by vaccination with r*Sm*10.3 was demonstrated by 25.5–32% reduction in the worm burden, 32.9–43.6% reduction in the number of eggs per gram of hepatic tissue, a 23.8% reduction in the number of granulomas, an 11.8% reduction in the area of the granulomas and a 39.8% reduction in granuloma fibrosis.

Conclusions/Significance: Our data suggest that *Sm*10.3 is a potential candidate for use in developing a multi-antigen vaccine to control schistosomiasis and provide the first evidence for a possible role for *Sm*10.3 in the blood feeding process.

Citation: Martins VP, Morais SB, Pinheiro CS, Assis NRG, Figueiredo BCP, et al. (2014) *Sm*10.3, a Member of the Micro-Exon Gene 4 (MEG-4) Family, Induces Erythrocyte Agglutination *In Vitro* and Partially Protects Vaccinated Mice against *Schistosoma mansoni* Infection. PLoS Negl Trop Dis 8(3): e2750. doi:10.1371/journal.pntd.0002750

Editor: John Pius Dalton, McGill University, Canada

Received October 6, 2013; **Accepted** February 4, 2014; **Published** March 20, 2014

Funding: This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES), INCT-Doenças Tropicais, and Pro-Reitoria de Pesquisa da UFMG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: scozeus@icb.ufmg.br

Introduction

Schistosomiasis occurs primarily in developing countries and is the most important human helminth infection in terms of global mortality. This parasitic disease affects more than 200 million people worldwide, causing more than 250,000 deaths per year [1]. Furthermore, schistosomiasis is responsible for the loss of up to 4.5 million DALYs (disability adjusted life years) annually [2]. Current schistosomiasis control strategies are mainly based on chemotherapy but, despite decades of mass treatment, the number of infected people has not decreased considerably in endemic areas [3–5]. The extent of endemic areas, constant reinfection of individuals and poor sanitary conditions in developing countries make drug treatment alone inefficient [6]. It is thought that the best long-term strategy for controlling schistosomiasis is through immunization with an anti-schistosome vaccine combined with drug treatment [7]. A vaccine that induces even a partial reduction in worm burdens could considerably reduce pathology and limit parasite transmission [8].

Currently, the most promising schistosome vaccine candidates are proteins located on the surface of the worms [9], such as the

Author Summary

Schistosomiasis mainly occurs in developing countries and is the most important human helminth infection in terms of global mortality. This parasitic disease affects more than 200 million people worldwide and causes more than 250,000 deaths per year. Current schistosomiasis control strategies are mainly based on chemotherapy, but many researchers believe that the best long-term strategy for controlling schistosomiasis is a combination of drug treatment and immunization with an anti-schistosome vaccine. Consequently, significant effort has been dedicated to developing and characterizing an anti-schistosome vaccine. Over the last five years, considerable data have been generated regarding the genomics, transcriptomics and proteomics of *Schistosoma mansoni*. In the present study, we characterize the *Sm*10.3 protein and evaluate its potential to protect against *S. mansoni* infection in a murine model. We demonstrate that *Sm*10.3 is primarily expressed during the stages of the parasite life cycle that involve infection and disease development in the human host. *Sm*10.3 is located on the surface of the digestive epithelia of adult female worms, an important host/parasite interface. Moreover, the vaccination of mice with r*Sm*10.3 confers partial protection against *S. mansoni*. Taken together, our data suggest that *Sm*10.3 may be a useful component of a multi-antigen vaccine against schistosomiasis.

tegument proteins TSP-2 [10] and *Sm*29 [11]. The tegument is a dynamic host-interactive surface involved in nutrition, immune evasion/modulation, excretion, osmoregulation, sensory reception, and signal transduction [12,13]. Other surface-exposed proteins with high potential as vaccine targets are located in the digestive tract of lung-stage schistosomula and adult worms [14–18].

In this study, we evaluated the potential of Sm10.3, a member of the micro-exon gene 4 (MEG-4) family, to serve as a component of a recombinant subunit vaccine. The *Sm*10.3 antigen was first described in 1988 [19], but the *Sm10.3* and MEG family genes were not completely characterized until the recent publication of the *S. mansoni* genome [20]. There are multiple copies of some MEGs in the *S. mansoni* genome, arranged as tandem, symmetrically organized exons with lengths that are a multiples of three bases (from 6 and 36 base pairs) [20,15]. It is thought that this arrangement may lead to protein variation through alternative splicing. Moreover, most of the MEGs are up-regulated during the stages in the parasite life cycle that involve establishment in the mammalian host [15].

In this study, we determined that *Sm*10.3 is located on the surface of the digestive tract of *S. mansoni* adult female worms and lung-stage schistosomula. We detected higher levels of *Sm10.3* mRNA in the schistosomula stage of the parasite life cycle. We also show preliminary evidence that *Sm*10.3 plays a role in erythrocyte agglutination. Furthermore, we report that vaccination with r*Sm*10.3 induces a mixed Th1/Th2-type immune response in mice, which correlates with a reduction in the worm burden and liver pathology.

Methods

Ethics statement

All animal experiments were conducted in accordance with the Brazilian Federal Law number 11.794, which regulates the scientific use of animals, and IACUC guidelines. All protocols were approved by the Committee for Ethics in Animal Experimentation (CETEA) at Universidade Federal de Minas Gerais UFMG under permit 179/2010.

Mice and parasites

Female C57BL/6 mice aged 6–8 weeks were purchased from the Federal University of Minas Gerais (UFMG) animal facility. *S. mansoni* (LE strain) cercariae were maintained in *Biomphalaria glabrata* snails at CPqRR (Centro de Pesquisa René-Rachou-Fiocruz) and prepared by exposing infected snails to light for 1 h to induce shedding. Cercarial numbers and viability were determined prior to infection using a light microscope.

*Sm*10.3 cloning and antigen preparation

The plasmid pJ414 containing the sequence for r*Sm*10.3 (pJ414::*Sm*10.3) was manufactured by DNA 2.0 (https://www.dna20.com) using DNA2.0 optimization algorithms for expression in *Escherichia coli*. This plasmid was transformed into *E. coli* Rosetta-gami (Merck KGaA, Darmstadt, Germany) competent cells. Transformants harboring the designed plasmid were screened on LB agar plates containing ampicillin (50 μg/ml) and cloranphenicol (34 μg/ml) and the selected transformant was designated as r*Sm*10.3-Rosetta. One liter of r*Sm*10.3-Rosetta was cultured in a three-liter erlenmeyer on a rotary shaker at 200 rpm at 37°C to an optical density at 600 nm of approximately 0.5–0.8 and gene expression was induced by using 1 mM isopropylthiogalactoside (IPTG). After 5 h of induction, the bacterial cells were harvested by centrifugation at 4,000× g for 20 min. Using gently vortexing or pipetting, the pellet was resuspended in 50 ml of 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl and 20 mM imidazole. Subsequently, the cells were submitted to three cycles of sonication lasting 30 s each and centrifuged at 5400× g for 20 min. The r*Sm*10.3 was recovered solubilized in the supernatant and purified by affinity chromatography on a Ni-Sepharose column (Hitrap chelating 5 mL) using an AKTA explorer chromatography system (GE Healthcare, São Paulo, Brazil). After protein binding to the Ni-Sepharose column, washes with 50 mM imidazole were performed and the protein was eluted with 500 mM imidazole. Fractions containing the protein were determined through Bradford's method (Coomassie Protein Assay Kit, Pierce) and also SDS/PAGE-12% and dialyzed against PBS pH 7.0. The dialysis was carried out at 4°C using a Spectra/Por2 membrane (MWCO 6 to 8 kDa; Spectrum Medical Industries, Inc., Laguna Hills, CA). The recombinant protein was quantified using the Bradford's method and used as antigen for vaccination and immunological experiments. All reagents were purchased from Sigma-Aldrich, CO (St. Louis, MO, USA) unless otherwise specified.

Real-time PCR

Total RNA was isolated from adult parasites, eggs, miracidia, cercariae or schistosomula using published procedures [21]. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were treated with DNAse, purified and concentrated using the RNeasy Micro Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. A 1.5 μg portion of each sample was reverse transcribed using the SuperScript*H* III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA). Specific primer pairs (5′-CTT AAT CAA TAA GCC AAA GG-3′ and 5′-TAT TGA TTT GTC GTA ATA GT-3′) were designed using the Primer Express program (Applied Biosystems, Foster City, CA, USA) and default parameters and arbitrarily named primers 1 and 2, respectively. Real-time RT-PCR reactions were conducted in

triplicate in a 20 µL volume containing 10 µL of Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 160 nmol of each primer (primers 1 and 2) and 0.30 µL of the cDNA generated by reverse transcription. Real-time RT-PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the following cycling parameters: 60°C for 10 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. A dissociation curve was generated using the following conditions: 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. Real-time data were normalized to the expression level of NADH dehydrogenase. *p*-values were determined by Student's *t*-test, using one-tailed distribution and heteroscedastic variance.

SDS-PAGE and immunoblotting

Purified r*Sm*10.3 was analyzed on 15% polyacrilamide SDS-PAGE gels prepared and run as previously described [22]. The gel was then transferred to a nitrocellulose membrane [23]. The membrane was blocked with TBS-T (0.5 M NaCl−0.02 M Tris (pH 7.5), 0.05% Tween 20) containing 5% dry milk for 16 h at 4°C. The membrane was then incubated with a mouse monoclonal antibody to the 6×His-tag (GE Healthcare, Pittsburgh, PA, USA) diluted 1:2,000 or a mouse polyclonal antibody to r*Sm*10.3 diluted 1:1,000 for 1 h at room temperature. After three washes with TBS-T, the membrane was incubated in 1:2,000 goat anti-mouse IgG conjugated to alkaline phosphatase (AP), then treated with a developing buffer containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP). After the membrane was imaged, it was washed with distilled water and dried by sandwiching between two sheets of filter paper for storage. All reagents were purchased from Sigma-Aldrich, CO (St. Louis, MO, USA) unless otherwise specified.

Immunization of mice

Six to eight week-old female C57BL/6 mice were divided into two groups of ten mice each. Mice were injected subcutaneously at the nape of the neck with 25 µg of r*Sm*10.3 protein or PBS (for the control group) on days 0, 15 and 30. The protein mixture was formulated using Complete Freund's Adjuvant (CFA) for the first immunization and Incomplete Freund's Adjuvant (IFA) for the last two immunizations (Sigma-Aldrich, CO, St. Louis, MO, USA).

Immunolocalization of *Sm*10.3 in *S. mansoni* adult and worms and lung-stage schistosomula

For the microscopy studies, adult worms were recovered from perfused mice, and lung-stage schistosomula were prepared *in vitro* as described by Harrop & Wilson [24]. Parasites were fixed in Omnifix II (Ancon Genetics, St Petersburg, FL, USA) for sectioning. For the sectioning assays, 7 µm slices of Paraffin-embedded adult male or female parasites were deparaffinized using xylol and hydrated with an ethanol series, [25]. For experiments using *in vitro* cultured lung-stage schistosomula, a whole-mount protocol was chosen, lung stage schistosomula were treated with permeabilizing solution (0.1% Triton X-100, 1% BSA and 0.1% sodium azide in PBS pH 7.2) overnight at 4°C [25]. Following, permeabilized schistosomula and parasite sections were blocked with 1% BSA (bovine serum albumin) in PBST (phosphate buffered saline, pH 7.2 with 0.05% Tween-20) for 1 h and incubated with anti-r*Sm*10.3 serum diluted 1:20 in blocking buffer. Serum from non-immunized mice was used as a negative control. The samples were washed three times with PBST and incubated with an anti-mouse IgG antibody conjugated to FITC (Molecular Probes, Carlsbad, CA, USA) diluted 1:100 in blocking buffer containing rhodamine phalloidin (Molecular Probes, Carlsbad, CA, USA) to stain the actin microfilaments. The samples were washed four times and mounted with ProLong Gold anti-fading mounting medium containing DAPI (Molecular Probes, Carlsbad, CA, USA). Schistosomula were imaged on a Nikon A1R confocal microscope (60× NA1.4-CFI-Plan-Apo oil objective), and adult worms were imaged on a Nikon Eclipse Ti microscope from the Microscopy Center of the Biological Sciences Institute (CEMEL) at the Federal University of Minas Gerais (UFMG). All reagents were purchased from Sigma-Aldrich, CO (St. Louis, MO, USA) unless otherwise specified.

Erythrocyte agglutination assays

Mice blood for the hemagglutination assays were collected and processed as described previously [26]. Blood was withdrawn from mice with a syringe, added to tubes containing EDTA (at a final concentration of 12 mM) and centrifuged for 15 min at 3000× g at room temperature. After centrifugation, the plasma was transferred to a fresh tube. In a second tube, erythrocytes were washed three times and suspended in PBS (pH 7.2) to a hematocrit of 20 or 40%. The erythrocyte suspensions were used for the agglutination assays on glass slides, in the hemagglutination endpoint dilution assays or in the hemagglutination kinetics assays.

Agglutination assays on glass slides was performed as previously described [26], the erythrocyte suspensions were prepared as above to a final hematocrit of 20% diluted in PBS. Next, 9 µl of each erythrocyte suspension was combined with 1 µl of r*Sm*10.3 containing 5 µg of purified protein, 1 µl of PBS or 1 µl of PBS containing 5 µg of r*Sm*29 (an unrelated *S. mansoni* antigen) as negative controls. Recombinant protein r*Sm*29 [11] was expressed with a 6×-histidine tag and purified in a similar way to r*Sm*10.3, as described in previous sections. The 10 µl mixture was loaded onto glass slides, covered with coverslips and immediately visualized using 10× and 40× objective lenses on a microscope equipped with a JVC TK-1270/RBG micro camera.

Hemagglutination endpoint dilution assays were performed as previously described [27,28]. A serial two-fold dilution of 50 µl of r*Sm*10.3, r*Sm*29 and Concanavalin A solutions (protein concentrations ranged from 500 µg/mL to 0.97 µg/mL) in microtiter U-plates was mixed with 50 µl of a 2% suspension of mice erythrocytes in PBS at 37°C, resulting in a final erythrocytes suspension volume of 100 µl and a hematocrit of 1%. In the blank wells 50 µl of a 2% suspension of mice erythrocytes were mixed with 50 µl of PBS. Concanavalin A was used as positive control of the hemagglutination process [29–31] and r*Sm*29 (an unrelated *S. mansoni* antigen) as negative control. The results were read after approximately 1 h when the blank had fully sedimented. The endpoint was defined as the highest dilution showing complete hemagglutination. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was defined as one hemagglutination unit. Specific activity is the number of hemagglutination units per mg of protein per milliliter [32].

Mice polyclonal antibodies raised against r*Sm*10.3 were used as inhibitor in hemagglutination inhibition assays according to a protocol previously described [33], with modifications. Twenty five microliters of r*Sm*10.3 solutions with three different protein concentrations (1000 µg/mL, 500 µg/mL and 250 µg/mL) were mixed with a serial two-fold dilution of 25 µl of anti- r*Sm*10.3 mice serum, ranging from 1:1 to 1:32 dilution and incubated at 37°C for 30 min. Following, 50 µl of a 2% suspension of mice erythrocytes in PBS was added to the wells and incubated for 1 h at 37°C.

The kinetics of the hemagglutination process were monitored by analyzing variations in turbidity [26]. Briefly, 100 µl of the erythrocyte suspension (in PBS) at a hematocrit of 5% was added to 96-well plates, and the agglutination was triggered by adding 0.5 to 5 µg of r*Sm*10.3 diluted in 100 µl of PBS, 100 µl of PBS containing 5 µg of Concanavalin A, as positive control of the hemagglutination process and 100 µl of PBS or 100 µl of PBS containing 5 µg of r*Sm*29 as negative controls, resulting in a final volume of 200 µl and a hematocrit of 2.5%. Plates were incubated at 37°C and spectrophotometric readings were taken at 655 nm every 13 s, with 8 s of shaking between each reading in a VersaMax Tunable Microplate reader (Molecular Devices, Sunnyvale, CA, USA). All reagents were purchased from Sigma-Aldrich, CO (St. Louis, MO, USA).

Infection and worm recovery

Fifteen days after the final immunization, the mice were challenged with 100 cercariae (LE strain) by percutaneous exposure of the abdominal skin for 1 h. Forty-five days after the challenge, the adult worms were perfused from the portal veins, as described previously [34]. Two independent experiments were performed to determine protection levels. The degree of protection was calculated by comparing the number of worms recovered from each vaccinated group to the respective control group, using the following formula:

$$\mathrm{PL} = \mathrm{WRCG} - \mathrm{WREG} \times 100/\mathrm{WRCG},$$

where PL indicates the protection level, WRCG indicates the number of worms recovered from the control group, and WREG indicates the number of worms recovered from the experimental group.

Measurement of specific anti-r*Sm*10.3 antibodies

Following immunization, sera were collected from ten mice in each experimental group at two week intervals. The levels of specific anti-*Sm*10.3 antibodies were measured by indirect ELISA. Maxisorp 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 µg/mL r*Sm*10.3 in carbonate-bicarbonate buffer (pH 9.6) for 16 hat 4°C, then blocked for 2 h at room temperature with 200 µL/well PBST (phosphate buffer saline, pH 7.2 with 0.05% Tween-20) plus 10% FBS (fetal bovine serum). One hundred microliters of serum diluted 1:100 in PBST was added to each well, and the plates were then incubated for 1 h at room temperature. Plate-bound antibodies were detected using peroxidase-conjugated anti-mouse IgG, IgG1 and IgG2a (Southern Biotechnology, CA, USA) diluted 1:10000, 1:5000 and 1:2000 in PBST, respectively. The color reaction was induced by adding 100 µL of 200 pmol OPD (o-phenylenediamine) in citrate buffer (pH 5.0) plus 0.04% H_2O_2 to each well for 10 min. The color reaction was stopped by adding 50 µL of 5% sulfuric acid to each well. The plates were read at 495 nm in an ELISA plate reader (BioRad, Hercules, CA, USA). All reagents were purchased from Sigma-Aldrich, CO (St. Louis, MO, USA) unless otherwise specified.

Cytokine analysis

The cytokine experiments were performed using cultured splenocytes from individual mice immunized with r*Sm*10.3 or PBS (n = 4 for each group). The splenocytes were isolated from the macerated spleens of individual mice one week after the third immunization and washed twice with sterile PBS. After washing, the cells counts were adjusted to 1×10^6 cells per well in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 100 U/mL of penicillin G sodium and 100 µg/mL of streptomycin sulfate. The splenocytes were maintained in culture with medium alone or stimulated with r*Sm*10.3 protein (15 µg/mL), concanavalin A (ConA) (5 µg/mL), or LPS (1 µg/mL), as previously described [34]. The 96-well plates (Nunc, Roskilde, Denmark) were maintained in a 37°C incubator with a 5% CO_2 atmosphere. The culture supernatants were collected after 24 h to measure IL-5 levels, after 48 h to measure TNF-α levels and after 72 h to measure IFN-γ and IL-10 levels. The cytokine measurement assays were performed using the DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All reagents were purchased from Sigma-Aldrich, CO (St. Louis, MO, USA) unless otherwise specified.

Histopathological analysis

Following perfusion to recover the schistosomes, liver samples were collected from 8 animals each from the control and experimental groups to evaluate the effect of immunization on granuloma formation. The liver samples, which were taken from the central part of the left lateral lobe, were fixed with 10% buffered formaldehyde in PBS. Histological sections were performed using microtome at 6 µm and stained on a slide with picrosirius-haematoxylin-eosin (PSHE). The count of granulomas was performed at a microscope with 10× objective lens. Each liver section was scanned for calculating its whole area (mm^2) using the ImageJ software (http://rsbweb.nih.gov/ij/index.html). For measurement of the total area of granulomas, a microscope with 10× objective lens was used; images were obtained through a JVC TK-1270/RBG microcamera attached to the microscope. Twenty granulomas with a single-well-defined egg were randomly selected, in each liver section and the granuloma area was measured using the ImageJ software. Granuloma fibrosis was analyzed using the software analysis getIT (Olympus Soft Imaging getIT) and images to illustrate the fibrosis area were edited using Adobe Photoshop software. All reagents were purchased from Sigma-Aldrich, CO (St. Louis, MO, USA).

Statistical analysis

The results from the two experimental groups were compared by Student's *t*-test using the software package GraphPad Prism (La Jolla, CA). Bonferroni adjustments were included for multiple comparisons. The *p*-values obtained by this method were considered significant if they were <0.05.

Accession numbers

*Sm*10.3 (M22346.1), *Sm*29 (AF029222.1), Tsp2 (AF521091.1).

Results

Expression profile of *Sm*10.3 at different stages in the *S. mansoni* life cycle

The expression of the *Sm10.3* gene was detected by real-time PCR at different stages in the *S. mansoni* life cycle. The only stage during which *Sm*10.3 mRNA was not detected was the miracidium stage. The highest level of *Sm*10.3 mRNA expression was observed in lung-stage schistosomula. *Sm*10.3 expression was also detected in eggs, adult worms and cercariae, but at lower levels than in the schistosomula (Fig. 1).

Expression and purification of r*Sm*10.3

Cloning and heterologous expression of the *Sm10.3* gene was performed as described in the material and methods section.

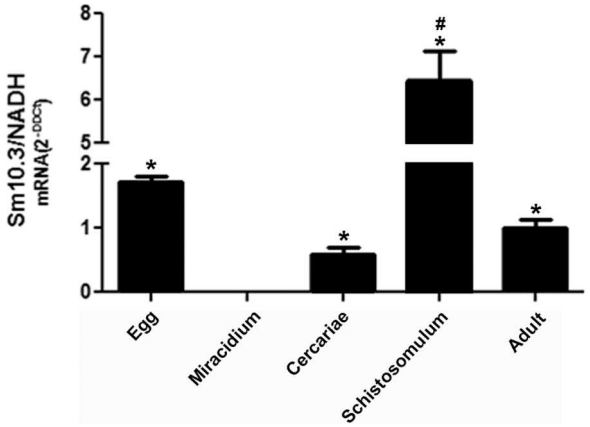


Figure 1. Expression profile of *Sm10.3* at different stages in the *S. mansoni* life cycle. Real time RT-PCR showing relative levels of *Sm10.3* transcripts at different stages in the *S. mansoni* life cycle (egg, miracidium, cercaria, schistosomulum and adult worm). Statistically significant differences compared to miracidia are denoted by asterisks, and statistically significant differences compared to eggs, cercariae and adult worms are indicated by # ($p<0.05$). Error bars indicate intra-assay standard deviation of means. Results are representative of two independent experiments.
doi:10.1371/journal.pntd.0002750.g001

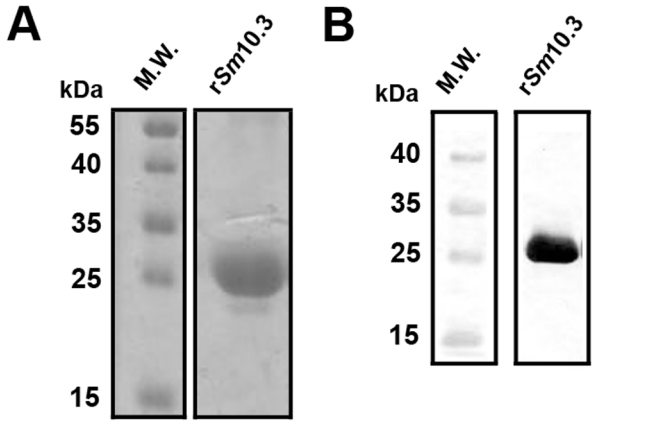


Figure 2. Heterologous expression and purification of r*Sm*10.3. (A) SDS-PAGE stained with Coomassie brilliant blue showing eluted and dialyzed r*Sm*10.3 after purification by Ni^{2+}-charged column chromatography. Ten microliters were loaded per lane. The molecular weight protein standard (M.W.) is a broad range pre-stained ladder from BioRad. (B) Western blot analysis of r*Sm*10.3 proteins probed with monoclonal mouse anti-His tag antibodies. Ten micrograms of proteins were loaded per lane. The molecular weight protein standard (M.W.) is a broad range pre-stained ladder from BioRad.
doi:10.1371/journal.pntd.0002750.g002

Recombinant *Sm*10.3 (r*Sm*10.3) was purified from bacteria, and the first seven fractions that were eluted from an affinity chromatography column were combined and dialyzed in PBS pH 7.2 and then analyzed by SDS-PAGE followed by Coomassie blue staining (Fig. 2A). The strong band visible at approximately 26 kDa, the expected mass of the purified r*Sm*10.3, indicates the success of the purification protocol (Fig. 2A). To further evaluate the specificity of the purification procedure, the purified r*Sm*10.3 was analyzed by western blot using a mouse monoclonal anti-His tag antibody (Fig. 2B). The western blot analysis confirmed that the protein around 26 kDa had a histidine tag and this is the molecular weight expected for r*Sm*10.3 (Fig. 2A, B).

*Sm*10.3 is localized on the surface of the intestinal tract of adult *S. mansoni*

The localization of *Sm*10.3 was determined in *S. mansoni* lung-stage schistosomula (Fig. 3C and D), female adult parasites (Fig. 3G and H) and male adult parasites (Fig. 3K and L) using specific mouse polyclonal antibodies to r*Sm*10.3 and fluorescence microscopy. Rhodamine phalloidin (red) was used as an actin marker to label the cytoskeletal tegument components and muscle layers (Fig. 3B, D, F, H, J and L). The cell nuclei were stained with DAPI (blue) (Fig. 3). The native *Sm*10.3 protein (green) was located exclusively in the internal tissues of lung-stage schistosomula (Fig. 3C and D), as well as on the surface of the esophageal and intestinal epithelia of adult parasites (Fig. 3G and H). No *Sm*10.3-specific signal was detected in sera from naïve mice (pre-serum) in either the adult parasites (Fig. 3E, F, I and J) or the lung-stage schistosomula (Fig. 3A and B).

r*Sm*10.3 induces agglutination of mouse erythrocytes

To evaluate the role of *Sm*10.3 in the digestive epithelia of adult worms and its possible contribution to the erythrocyte feeding process, we analyzed the effect of r*Sm*10.3 on mouse erythrocytes suspended in PBS. The kinetics of the hemagglutination process were monitored analyzing variations in turbidity by an adapted protocol that was previously described [26]. This methodology allows the measurement of hemagglutination due to the formation of erythrocyte clumps and a subsequent reduction in absorbance. Different amounts of r*Sm*10.3 were added to erythrocyte suspensions at a haematocrit of 5% and followed over time by reading the absorbance at 655 nm with a spectrophotometer. The recombinant protein r*Sm*29 [11] was used as a negative control and the lectin concanavalin A as a positive control of the erythrocytes hemagglutination process, as previously demonstrated [29–31]. The addition of a similar amount of r*Sm*29 did not induce any changes in the absorbance. However, the addition of 5 μg/mL or 10 μg/mL of r*Sm*10.3 resulted in a 5% reduction in absorbance, and higher amounts of r*Sm*10.3 (50 μg/mL) reduced the absorbance by 10–15% as compared to r*Sm*29, while 50 μg/mL of concanavalin A decreased the absorbance by 28–32% after 400 s compared to r*Sm*29 (Fig. 4I). The hemagglutination process induced by r*Sm*10.3 can also be observed microscopically, as shown by the formation of erythrocytes clumps upon the addition of recombinant *Sm*10.3 (Fig. 4E and F).

Later, the hemagglutinating effect of r*Sm*10.3 in mice erythrocytes was clearly evidenced by hemagglutination endpoint dilution assays. Protein concentrations of r*Sm*29 up to 250 μg/mL were not able to induce hemagglutination (Fig. 4G), while r*Sm*10.3 caused erythrocytes agglutination with the lowest protein concentration of 31.2 μg/mL (Fig. 4G). The minimal protein concentration of concanavalin A required to induce hemagglutination was 3.9 μg/mL, approximately eight times lower than the amount observed for r*Sm*10.3 (Fig. 4G). These results confer to concanavalin A a total hemagglutinating activity of 1.56×10^2 units (U) and a specific hemagglutinating activity of 40000 U/mg/mL (Table S1), while these values for r*Sm*10.3 were 13 times lower and 100 times lower, respectively, 0.12×10^2 units and 400 U/mg/mL (Table S1).

By means of hemagglutination inhibition assays using anti-r*Sm*10.3 it was demonstrated the specific and protein concentration dependent pattern of the r*Sm*10.3 hemagglutinating activity (Fig. 4H). There was no hemagglutination inhibition with 250 μg/mL of r*Sm*10.3, even at the highest anti-r*Sm*10.3 concentration (1:1 dilution). However, with lower r*Sm*10.3 concentrations its hemagglutinating activity was inhibited by anti-r*Sm*10.3 (Fig. 4H). When

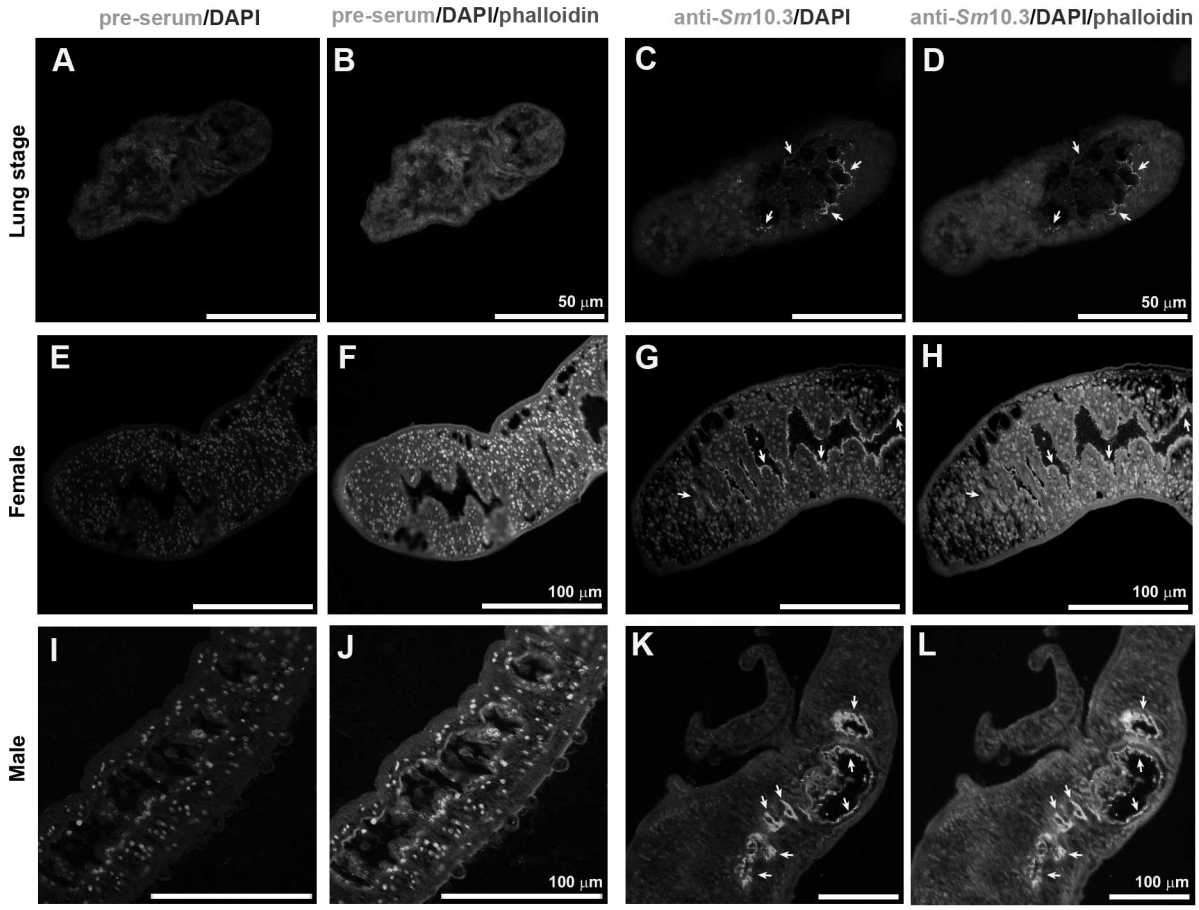


Figure 3. Immunolocalization of *Sm*10.3 in *S. mansoni* adult worms and lung-stage schistosomula. Mouse polyclonal anti-r*Sm*10.3 and anti-mouse secondary antibodies coupled to Alexa 488 (green) were used to label the native *Sm*10.3 protein (C, D, G, H, K, L). Serum from naive mice was used as a negative control (A, B, E, F, I, J). Nuclei were stained with DAPI (blue), and actin was stained with phalloidin conjugated to rhodamine (red). White arrows indicate the localization of *Sm*10.3 in the internal tissues of the schistosomula and on the surface and lumen of the esophageal and intestinal tegument of adult worms. Schistosomula are shown as maximum Z projections of 3 planes imaged with 1.0 μm distance intervals and adult parasites are single scans.
doi:10.1371/journal.pntd.0002750.g003

using 125 μg/mL of r*Sm*10.3 there was inhibition at anti-r*Sm*10.3 dilutions ranging from 1:1 to 1:8, while with 62.5 μg/mL of r*Sm*10.3 the inhibition was evident from 1:1 to 1:16 dilutions (Fig. 4H).

Antibody profile following the immunization of mice with r*Sm*10.3

Sera from ten animals from each vaccination group were tested by ELISA to evaluate the levels of specific IgG, IgG1 and IgG2a antibodies to rSm10.3. Significant titers of anti-r*Sm*10.3 IgG antibodies were detected at all time points tested after the first immunization (Fig. 5A). The levels of specific IgG1 antibodies increased at 30 days after the first immunization (Fig. S1A), while the levels of specific IgG2a antibodies continued to increase up to day 75 (Fig. S1B). Furthermore, the IgG1/IgG2a ratio was reduced at days 45, 60, 75 and 90 (Fig. 5B), which correlates with the elevation in anti-r*Sm*10.3 IgG2a production (Fig. S1B).

Immunization with r*Sm*10.3 induces protective immunity in mice

To test the potential usefulness of r*Sm*10.3 as part of an anti-schistosome vaccine, we asked whether this recombinant antigen could induce protection in a murine model of *S. mansoni* infection. Two independent vaccination trials were conducted and C57BL/6 mice were immunized three times with r*Sm*10.3 formulated with Freund's adjuvant and then challenged with 100 *S. mansoni* cercariae. The control group received adjuvant only in phosphate-buffered saline. Mice vaccinated with r*Sm*10.3 showed a 32.0% reduction in the adult worm burden in the first trial (Fig. 5C) and 25.5% reduction in the second trial (Fig. 5D). Regarding the number of eggs in mice livers, it was observed 43.6% and 32.9% reduction in the number of eggs per gram of liver tissue in the first and second trials, respectively (Fig. 5E).As shown in Figure 6A–D, histopathological analysis of the hepatic tissue, from animals of the first vaccination trial, demonstrated that r*Sm*10.3 immunization reduced the extent of fibrosis compared to control animals. These analysis showed a 23.8% reduction in liver granuloma counts (Fig. 6E), an 11.8% reduction in granuloma area (Fig. 6F), and a 39.8% reduction in granuloma fibrosis (Fig. 6G), as compared to control mice.

r*Sm*10.3 immunization induces a mixed Th1/Th2 cytokine profile

To evaluate the cytokine profile of mice immunized with r*Sm*10.3, splenocytes were isolated from spleens of vaccinated and

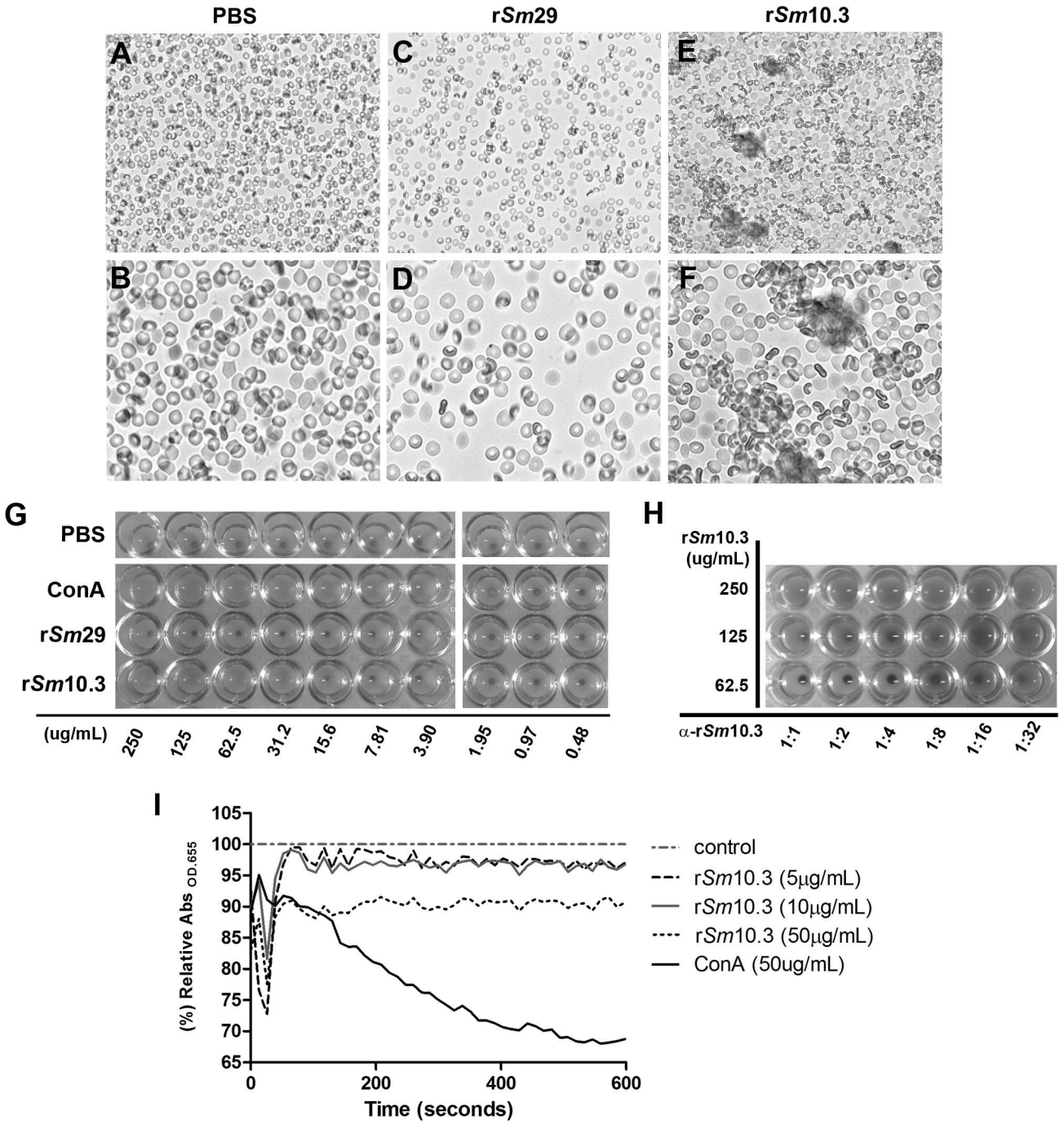


Figure 4. Erythrocyte agglutination assays to evaluate the hemagglutinating activity of r*Sm*10.3. *Microscopic imaging of the hemagglutinating activity of rSm10.3* (A–F). Erythrocyte suspensions (hematocrit of 20%) was combined with PBS (A, B), or 5 μg of r*Sm*29 (C, D), or 5 μg of purified r*Sm*10.3 (E, F). The mixture was loaded onto glass slides, covered with coverslips and immediately visualized at 40× (A, C, E) and 100× (B, D, F) magnification. The data shown are representative of three independent experiments. *Hemagglutination endpoint dilution assays* (G). Protein concentrations from 0.48 to 250 μg/mL of r*Sm*29, r*Sm*10.3 and concanavalin A (ConA) were tested in erythrocyte suspension (hematocrit of 1%). The deposition of erythrocytes onto the bottom of the wells, as a red dot, indicates a negative hemagglutination reaction, while the absence of erythrocytes deposition means a positive hemagglutination reaction. *Hemagglutination inhibition assays* (H). Three concentrations of r*Sm*10.3 (known to be sufficient to induce hemagglutination) were tested against six different dilutions of anti-r*Sm*10.3 antibodies, from 1:1 to 1:32 in a hematocrit of 1%. The deposition of erythrocytes onto the bottom of the wells indicates that the amount of anti-r*Sm*10.3 was sufficient to block r*Sm*10.3 activity, while the absence of erythrocytes deposition means that the amount of anti-r*Sm*10.3 was below the minimal concentration required to inhibit the r*Sm*10.3 hemagglutinating effect. *Kinetics of the hemagglutination process induced by rSm10.3* (I). Different concentrations of r*Sm*10.3 (5 μg/mL, 10 μg/mL and 50 μg/mL) were added to mouse erythrocytes at a final hematocrit of 2.5%. Fifty micrograms per mililiter of r*Sm*29 was used as negative control and 50 μg/mL of ConA was used as positive control. The absorbance values for r*Sm*29 were set as 100% and the absorbance readings for r*Sm*10.3 and ConA were compared to this standard. The data shown are representative of three independent experiments.
doi:10.1371/journal.pntd.0002750.g004

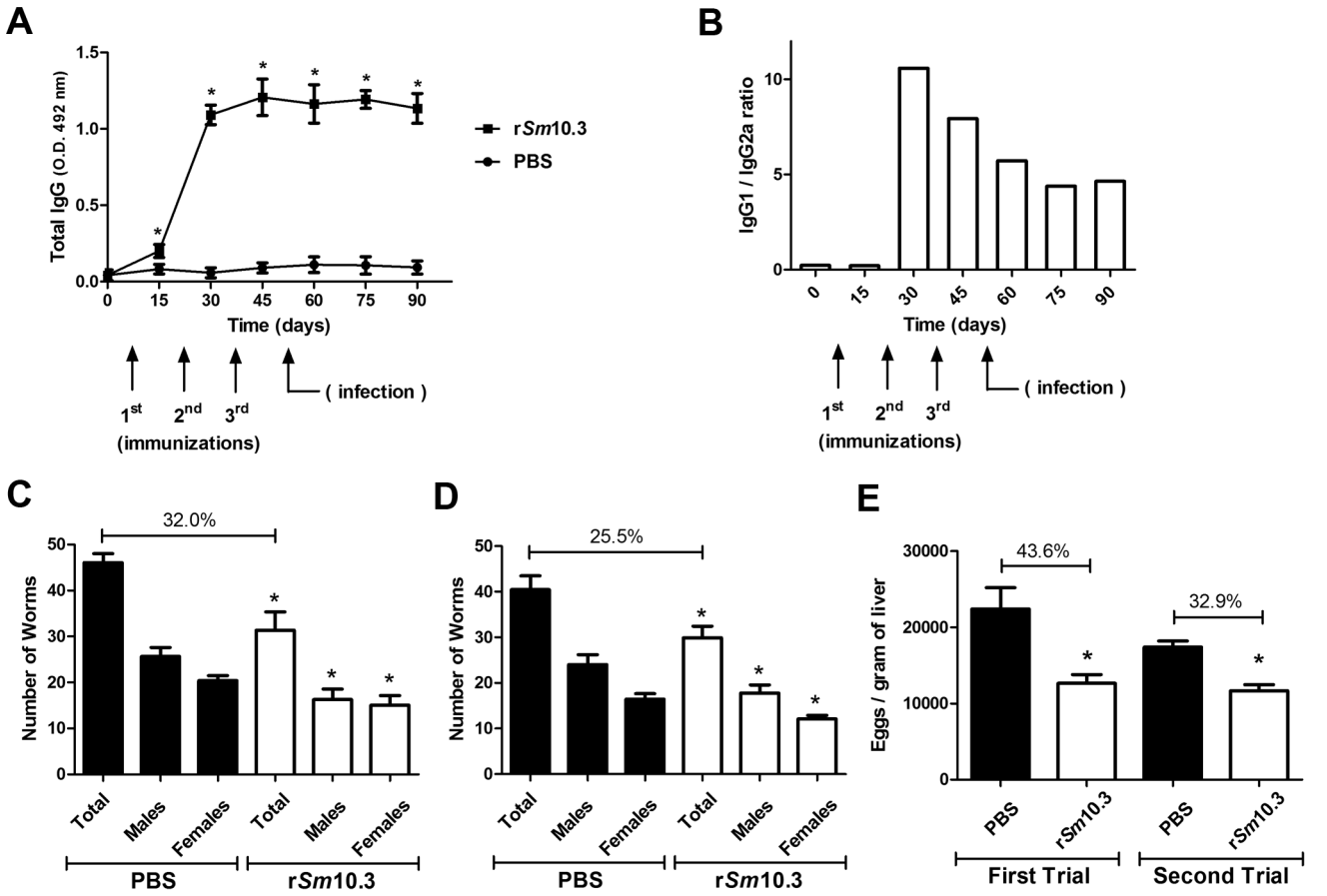


Figure 5. Vaccination with r*Sm*10.3 induces protective immunity in mice. (A), Sera from ten animals from each vaccination group were tested by ELISA to evaluate the levels of specific IgG antibodies to r*Sm*10.3. Significant titers of anti-r*Sm*10.3 IgG antibodies were detected at all time points tested after the first immunization. Arrows indicate when the three immunizations were administered and the infection was performed. The results shown are representative of two independent experiments. (B), IgG1 and IgG2a were tittered by ELISA as described in the material and methods and their values were used to calculate the IgG1/IgG2a ratio. Arrows indicate when the three immunizations were administered and the infection was performed. (C, D), Worm burden from two independent experiments of mice immunized with r*Sm*10.3 and challenged with live *S. mansoni* cercariae. Mice vaccinated with r*Sm*10.3 showed a 32.0% reduction in the adult worm burden in the first trial and 25.5% reduction in the second trial. (E), Oogram showing 43.6% and 32.9% reduction in the number of eggs per gram of liver tissue in the first and second trials, respectively. Error bars indicate intra-assay standard deviation of means. Asterisks indicate statistically significant differences between the vaccinated groups and the control group ($p<0.05$).
doi:10.1371/journal.pntd.0002750.g005

control animals after the third immunization. Statistically significant levels of IFN-γ, the signature cytokine of a Th1-type immune response, and TNF-α, a pro-inflammatory cytokine, were detected in the supernatant of cultured splenocytes from immunized animals compared to the control group (Fig. 7A and B, respectively). IL-5, a characteristic Th2-type cytokine, was also detected in the supernatant of cultured splenocytes from r*Sm*10.3-immunized mice at statistically significant levels compared to the PBS control (Fig. 7-D). Furthermore, high levels of the modulatory cytokine IL-10 were also observed in vaccinated animals (Fig. 7C). Concanavalin A (ConA) and LPS were used as positive controls to confirm that the splenocytes were responsive to stimuli. As shown in Figure 7, ConA induced the production of IFN-γ, IL-5 and IL-10, while LPS induced the production of TNF-α.

Discussion

Schistosomiasis is one of the most important neglected tropical diseases. Effective control is unlikely in the absence of improved sanitation and the development of a vaccine. Proteins located at the host/parasite interface, particularly molecules that are secreted or surface-exposed on the tegument and the intestinal epithelia, are the most promising targets for developing an anti-schistosomiasis vaccine [18]. We therefore evaluated the potential of the *Sm*10.3 antigen as a vaccine candidate, as it was previously reported to be localized on the esophageal gland in schistosomula and adult worms [15], as well as on the gut primordium of the nonfeeding cercaria [15]. The deduced amino acid sequence of *Sm10.3* contains a signal peptide, and the protein was predicted to be secreted or localized to the exterior surface of the cell. The gene products of several other MEG family members contain signal peptides for secretion and are secreted from different schistosomal glands and epithelia [20,15].

We confirmed previous reports [20,15] that *Sm10.3* is mainly expressed in the schistosomulum stage, as well as in other stages that involve contact with the mammalian host, such as eggs, cercariae and adult worms. MEG genes are difficult to clone, primarily due to extensive alternative splicing that generates variant transcripts of different sizes through exon skipping and the arbitrary combination of exons [19,20,15]. This variation in MEG gene products may represent a strategy used by members of the

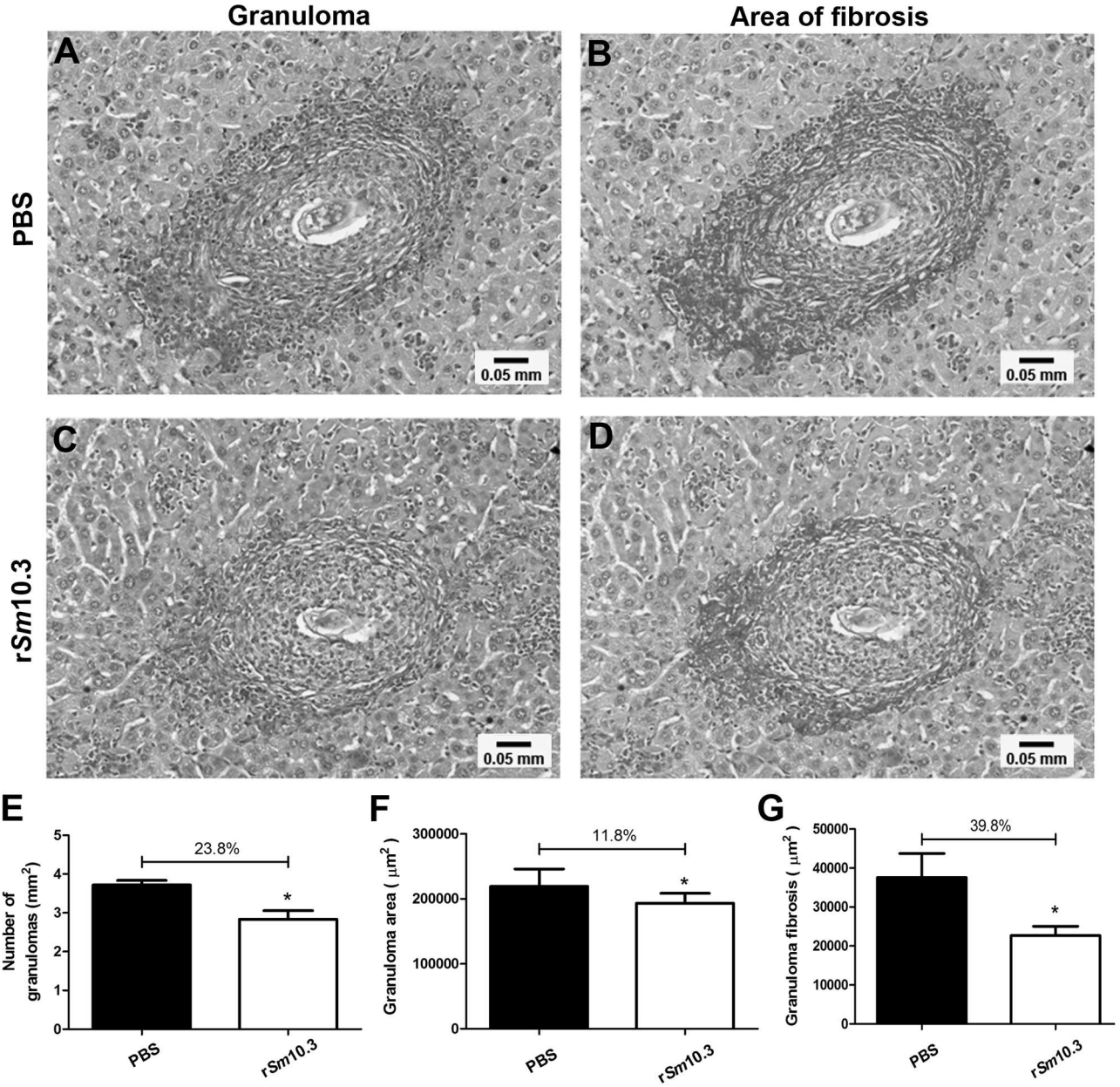


Figure 6. Histological analysis of hepatic tissue from mice vaccinated with r*Sm*10.3. The r*Sm*10.3-vaccinated and control animals from the first vaccination trial were sacrificed, and their livers were washed with PBS and stored in formaldehyde until sectioning and staining with picrosirius. (A) A representative sample from the PBS control group. (C) A representative sample from a mouse vaccinated with r*Sm*10.3. (B and D) Images were edited using image software to highlight the fibrotic areas in red. The images were captured using a 40× objective lens. (E), Graph showing a 23.8% reduction in the number of granulomas in vaccinated mice. (F), Graph showing a 11.8% reduction in the granuloma area in vaccinated mice. (G), Graph showing a 39.8% reduction in the granuloma fibrosis in vaccinated mice. Twenty granulomas with a single-well-defined egg were randomly selected in each liver section for the granuloma analysis. Error bars indicate intra-assay standard deviation of means. Asterisks indicate statistically significant differences between the vaccinated groups and the control group ($p<0.05$).
doi:10.1371/journal.pntd.0002750.g006

Schistosoma genus to confuse the host immune system, similar to the mechanisms of surface protein variation in *Trypanosoma brucei* and *Plasmodium falciparum* [20,15,18]. In this study, we produced the recombinant *Sm*10.3 protein from a synthetic gene that allowed us to express a protein corresponding to the largest transcript from the *Sm10.3* gene to optimize codon usage and avoid errors in the amino acid sequence.

Our fluorescence microscopy data confirm the *in silico* prediction that *Sm*10.3 is secreted or located on the cell surface, demonstrating that the native *Sm*10.3 protein localizes to the epithelia and lumen of the intestinal tract in adult parasites, as well as to the internal tissues of lung-stage schistosomula. A previous microarray study demonstrated an increase in *Sm10.3* expression in lung-stage schistosomula [15]. In the same study, *Sm*10.3 localization was examined using an antibody against a synthetic peptide (immunocytochemistry) and RNA hybridization (WISH). The authors found *Sm*10.3 proteins in the esophageal gland and the esophageal lumen of adult worms, but were unable to define the localization of *Sm*10.3 in the larval stage [15]. A more recent study from this group demonstrated that the *Sm*10.3 antigen is

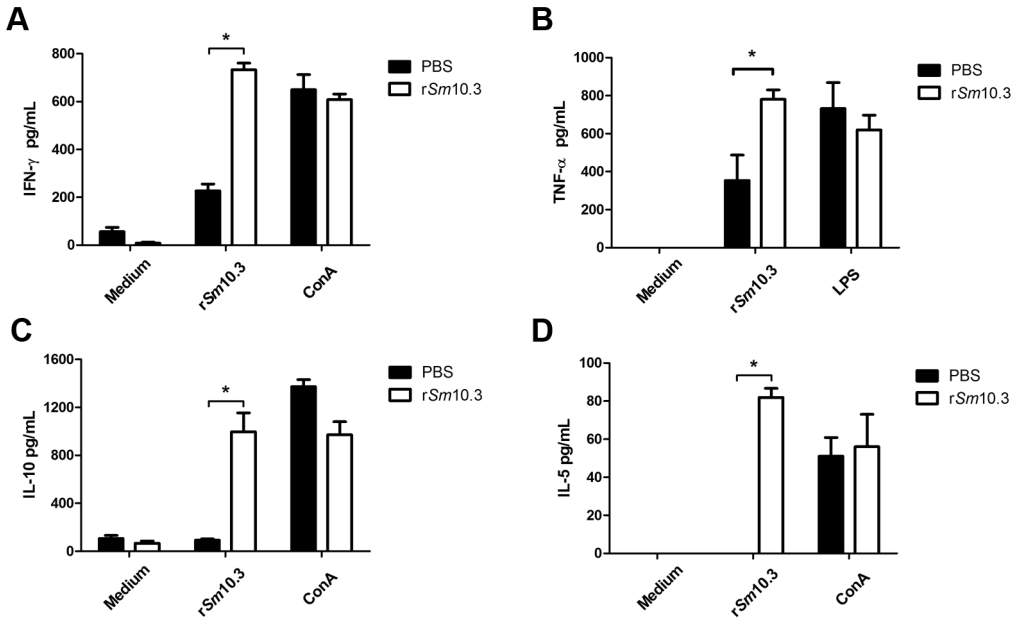


Figure 7. Cytokine profile of mice immunized with r*Sm*10.3. One week after the final immunization with r*Sm*10.3, splenocytes from five mice were isolated and assayed for IFN-γ (A), TNF-α (B), IL-10 (C) and IL-5 (D) production in response to medium, r*Sm*10.3 (25 μg/ml), ConA and LPS, as indicated. The control group was immunized with PBS plus Freund's adjuvant (CFA/IFA). The results are presented as the mean ± SD for each group. A statistically significant difference between the cytokines produced by splenocytes from the r*Sm*10.3-vaccinated mice and the control group is denoted by an asterisk ($p<0.05$). The results shown are representative of three independent experiments.
doi:10.1371/journal.pntd.0002750.g007

highly O-glycosylated, which means that native *Sm*10.3 a very sticky macromolecule that is highly likely to adhere to surfaces [35]. The authors suggest that this adherence could be responsible for the immunodetection of *Sm*10.3 not only in the esophageal gland but also throughout the entire esophagus (distal to secretion sites) [35,15]. We did not detect any *Sm*10.3 accumulation in the esophageal glands, but we did find *Sm*10.3 on the esophageal epithelia, in the esophageal lumen and in the gut epithelia. It was observed immunoreactivity in the gut epithelia and sometimes in the gut lumen in all samples that were imaged. However, it was not possible to obtain image sections showing the entire gut length, which prevent us to state that *Sm*10.3 is present in the whole gut. These apparent differences in *Sm*10.3 localization could be due to the different antibodies that were used in the studies. Alternatively, variant *Sm*10.3 transcripts could be differentially regulated depending on the experimental conditions. It has been previously shown that environmental stimuli can affect gene expression in *S. mansoni*, as the presence or absence of erythrocytes altered the transcription levels of genes expressed in the tegument and related to feeding [14].

To further investigate the role of *Sm*10.3 protein in the esophagus and gut of adult worms, we analyzed the effect of r*Sm*10.3 protein on erythrocytes and found that r*Sm*10.3 induced hemagglutination *in vitro*. This effect could be related to erythrocyte digestion and nutrition in adult worms, and may represent a role for *Sm*10.3 in adult worms. However, it is necessary to evaluate *in vivo* the impact of this *Sm*10.3-induced hemagglutination on the blood feeding process. In addition to the possible role of *Sm*10.3 protein in blood feeding, it is also likely that *Sm*10.3 contributes to protein variation, a role that has been previously proposed for the MEG family members [20,15,18]. The digestive tracts of schistosomes in the blood feeding stages are accessible to macromolecules such as albumin and immunoglobulins [36,37], which implies that there may be direct contact between the digestive epithelia and the host immune system.

We also assessed the interaction of *Sm*10.3 with the host immune system and its potential as a vaccine candidate. r*Sm*10.3 induced high levels of anti-r*Sm*10.3 IgG production in the sera of immunized mice after the second immunization. A decrease in the ratio between the IgG subtypes (IgG1/IgG2a) was observed 45 days after the first immunization. Furthermore, cytokine analysis of the supernatants of cultured splenocytes stimulated with r*Sm*10.3 suggests a mixed Th1/Th2-type immune response. Studies using the irradiated cercariae model, which induces high levels of protection in mice, suggest that effective protection can be based on a mixed Th1/Th2 response, a polarized Th1 response or even a polarized Th2 response [38]. In previous studies performed by our group, the majority of *S. mansoni* antigens tested as recombinant protein vaccines that conferred partial protection against cercariae challenges induced a Th1-type immune response [11,34,39,40] or a mixed Th1/Th2 response [41,42,43]. IFN-γ is involved in protective immunity against schistosomiasis, as specific anti-IFN-γ antibodies completely abolish the protection conferred by vaccination with irradiated cercariae [44]. Similar results were obtained in a study using IFN-γ knockout mice [45]. The partial protection conferred by vaccination with r*Sm*10.3 resulted in 25.5% to 32% reduction in worm burden, and the overall pathology was reduced, as shown by 32.9% to 43.6% reduction in the number of eggs per gram of hepatic tissue, a 23.8% reduction in the number of granulomas, an 11.8% reduction in the area of the granulomas and a 39.8% reduction in granuloma fibrosis. It is possible that the reduced liver pathology is related to the elevated levels of IL-10 detected in immunized mice, which may regulate Th2 responses and/or prevent the development of a polarized Th1 response, consequently reducing inflammation and liver injury [46,47].

In conclusion, our results confirm that *Sm*10.3 is mainly expressed during the stages of the *Schistosoma mansoni* life cycle that involve contact with the mammalian host. We show that the *Sm*10.3 protein is located in the intestinal tract of adult worms,

providing the first evidence for a possible role for *Sm*10.3 in the blood feeding process. Finally, our data suggest that *Sm*10.3 is a potential candidate for use in developing a multi-antigen vaccine to control schistosomiasis.

Supporting Information

Figure S1 Kinetics of specific IgG1 and IgG2a anti-r*Sm*10.3 production in the sera of vaccinated mice. Sera from 10 immunized mice per group were collected prior to the first immunization and at days 15, 30, 45, 60, 75 and 90 after the first immunization. The control group was injected with PBS plus Freund's adjuvant. The sera were assayed by ELISA for specific IgG1 and IgG2a antibodies. Arrows indicate when the three immunizations were administered. The results are presented as the mean absorbance at 492 nm for each group. Asterisks indicate statistically significant differences between the vaccinated groups and the control group ($p<0.05$). Error bars indicate intra-assay standard deviation of means. The results shown are representative of two independent experiments.
(TIF)

Table S1 Hemagglutinating activity of r*Sm*10.3 in mouse erythrocytes. The results were read after approximately 1 h when the blank had fully sedimented (See Fig. 4G). The endpoint was defined as the highest dilution showing complete hemagglutination. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was defined as one hemagglutination unit. Specific activity is the number of hemagglutination units per mg of protein per milliliter.
(PDF)

Author Contributions

Conceived and designed the experiments: VPM SCO. Performed the experiments: VPM SBM CSP NRGA BCPF NDR. Analyzed the data: VPM SBM CSP NRGA BCPF NDR SCO JAS. Contributed reagents/materials/analysis tools: MVC SCO. Wrote the paper: VPM SCO.

References

1. van der Werf MJ, de Vlas SJ, Brooker S, Looman CW, Nagelkerke NJ, et al. (2003) Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. Acta Trop 86: 125–139.
2. Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J (2006) Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect Dis 6: 411–425.
3. Harder A. (2002) Chemotherapeutic approaches to trematodes (except schistosomes) and cestodes: current level of knowledge and outlook. Parasitology Research 88: 587–590.
4. Mahmoud A, Zerhouni E. Neglected tropical diseases: moving beyond mass drug treatment to understanding the science. (2009) Health Aff (Millwood) 28:1726–33.
5. Gryssels, Schistosomiasis B. (2012) Infect Dis Clin North Am. 26:383–97.
6. Bergquist NR, Colley DG (1998) Schistosomiasis vaccine:research to development. Parasitol Today 14: 99–104.
7. Bergquist NR (2002) Schistosomiasis: from risk assessment to control. Trends Parasitol 18: 309–314.
8. Chitsulo L, Loverde P, Engels D (2004) Schistosomiasis. Nat Rev Microbiol 2: 12–13.
9. Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A (2010) Developing vaccines to combat hookworm infection and intestinal schistosomiasis. Nat Rev Microbiol 8: 814–826.
10. Tran MH, Pearson MS, Bethony JM, Smyth DJ, Jones MK, et al. (2006) Tetraspanins on the surface of Schistosoma mansoni are protective antigens against schistosomiasis. Nat Med 12: 835–840.
11. Cardoso FC, Macedo GC, Gava E, Kitten GT, Mati VL, et al. (2008) Schistosoma mansoni tegument protein Sm29 is able to induce a Th1-type of immune response and protection against parasite infection. PLoS Negl Trop Dis 2: e308.
12. Jones MK, Gobert GN, Zhang L, Sunderland P, McManus DP (2004) The cytoskeleton and motor proteins of human schistosomes and their roles in surface maintenance and host-parasite interactions. Bioessays 26: 752–765.
13. Van Hellemond JJ, Retra K, Brouwers JF, van Balkom BW, Yazdanbakhsh M, et al. (2006) Functions of the tegument of schistosomes: clues from the proteome and lipidome. Int J Parasitol 36: 691–699.
14. Gobert GN, Tran MH, Moertel L, Mulvenna J, Jones MK, et al. (2010) Transcriptional changes in Schistosoma mansoni during early schistosomula development and in the presence of erythrocytes. PLoS Negl Trop Dis 4: e600.
15. DeMarco R, Mathieson W, Manuel SJ, Dillon GP, Curwen RS, et al. (2010) Protein variation in blood-dwelling schistosome worms generated by differential splicing of micro-exon gene transcripts. Genome Res 20: 1112–1121.
16. Nawaratna SS, McManus DP, Moertel L, Gobert GN, Jones MK (2011) Gene Atlasing of digestive and reproductive tissues in Schistosoma mansoni. PLoS Negl Trop Dis 5: e1043.
17. Hall SL, Braschi S, Truscott M, Mathieson W, Cesari IM, et al. (2011) Insights into blood feeding by schistosomes from a proteomic analysis of worm vomitus. Mol Biochem Parasitol 179: 18–29.
18. Wilson RA (2012) Proteomics at the schistosome-mammalian host interface: any prospects for diagnostics or vaccines? Parasitology 139: 1178–1194.
19. Davis RE, Davis AH, Carroll SM, Rajkovic A, Rottman FM (1988) Tandemly repeated exons encode 81-base repeats in multiple, developmentally regulated Schistosoma mansoni transcripts. Mol Cell Biol 8: 4745–4755.
20. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, et al. (2009) The genome of the blood fluke Schistosoma mansoni. Nature 460: 352–358.
21. Hoffmann KF, Johnston DA, Dunne DW (2002) Identification of Schistosoma mansoni gender-associated gene transcripts by cDNA microarray profiling. Genome Biol. 3(8): e0041.
22. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
23. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76: 4350–4354.
24. Harrop R, Wilson RA (1993) Protein synthesis and release by cultured schistosomula of Schistosoma mansoni. Parasitology 107 (Pt 3): 265–274.
25. Drummond MG, Calzavara-Silva CE, D'Astolfo DS, Cardoso FC, et al. (2009) Molecular characterization of the Schistosoma mansoni zinc finger protein SmZF1 as a transcription factor. PLoS Negl Trop Dis. 10:e547.
26. Araujo RN, Pereira MH, Soares AC, Pereira ID, Diotaiuti L, et al. (2009) Effect of intestinal erythrocyte agglutination on the feeding performance of Triatoma brasiliensis (Hemiptera: Reduviidae). J Insect Physiol 55: 862–868.
27. Ng TB, Yu YL, Chu KT (2002) Isolation of a novel legumin-like lectin with potent hemagglutinating activity from seeds of the Chinese chestnut Castanea mollisima. Comp Biochem Physiol C Toxicol Pharmacol. 133:453–60.
28. Konozy EH, Bernardes ES, Rosa C, Faca V, et al. (2003) Isolation, purification, and physicochemical characterization of a D-galactose-binding lectin from seeds of Erythrina speciosa. Arch Biochem Biophys. 15:222–9.
29. Salit IE, Gotschlich EC (1977) Hemagglutination by purified type I Escherichia coli pili. J Exp Med. 1:1169–81.
30. Greig RG, Brooks DE (1979) Shear-induced concanavalin A agglutination of human erythrocytes. Nature. 13:738–9.
31. Greig RG, Brooks DE. (1981) Enhanced concanavalin A agglutination of trypsinised erythrocytes is due to a specific class of aggregation. Biochim Biophys Acta. 6:410–5.
32. Wang HX, Ng TB, Liu WK, Ooi, et al. (1995) Isolation and characterization oftwo distinct lectins with antiproliferative activity from the cultured mycelium ofthe edible mushroom *Tricholoma mongolicum*. Int J Pept Protein Res 46: 508–513.
33. Noah DL, Hill H, Hines D, White EL, et al. (2009) Qualification of the hemagglutination inhibition assay in support of pandemic influenza vaccine licensure. Clin Vaccine Immunol.16:558–66
34. Fonseca CT, Brito CF, Alves JB, Oliveira SC (2004) IL-12 enhances protective immunity in mice engendered by immunization with recombinant 14 kDa Schistosoma mansoni fatty acid-binding protein through an IFN-gamma and TNF-alpha dependent pathway. Vaccine 22: 503–510.
35. Li XH, de Castro-Borges W, Parker-Manuel S, Vance GM, Demarco R, et al. (2013) The schistosome oesophageal gland: initiator of blood processing. PLoS Negl Trop Dis 7: e2337.
36. Holtfreter MC, Loebermann M, Frei E, Riebold D, Wolff D, et al. (2010) Schistosomula, pre-adults and adults of Schistosoma mansoni ingest fluorescence-labelled albumin in vitro and in vivo: implication for a drug-targeting model. Parasitology 137: 1645–1652.
37. Thors C, Jokiranta TS, Meri T, Kairemo K, Meri S, et al. (2006) Immunoglobulin uptake and processing by Schistosoma mansoni. Parasite Immunol 28: 421–428.
38. Hewitson JP, Hamblin PA, Mountford AP (2005) Immunity induced by the radiation-attenuated schistosome vaccine. Parasite Immunol 27: 271–280.
39. Farias LP, Cardoso FC, Miyasato PA, Montoya BO, Tararam CA, et al. (2010) Schistosoma mansoni Stomatin like protein-2 is located in the tegument and induces partial protection against challenge infection. PLoS Negl Trop Dis 4: e597.
40. Garcia TC, Fonseca CT, Pacifico LG, Duraes Fdo V, Marinho FA, et al. (2008) Peptides containing T cell epitopes, derived from Sm14, but not from paramyosin,

induce a Th1 type of immune response, reduction in liver pathology and partial protection against Schistosoma mansoni infection in mice. Acta Trop 106: 162–167.
41. Pacifico LG, Fonseca CT, Chiari L, Oliveira SC (2006) Immunization with Schistosoma mansoni 22.6 kDa antigen induces partial protection against experimental infection in a recombinant protein form but not as DNA vaccine. Immunobiology 211: 97–104.
42. Pacheco LG, Zucconi E, Mati VL, Garcia RM, Miyoshi A, et al. (2005) Oral administration of a live Aro attenuated Salmonella vaccine strain expressing 14-kDa Schistosoma mansoni fatty acid-binding protein induced partial protection against experimental schistosomiasis. Acta Trop 95: 132–142.
43. Martins VP, Pinheiro CS, Figueiredo BC, Assis NR, Morais SB, et al. (2012) Vaccination with enzymatically cleaved GPI-anchored proteins from Schistosoma mansoni induces protection against challenge infection. Clin Dev Immunol 2012: 962538.
44. Smythies LE, Coulson PS, Wilson RA (1992) Monoclonal antibody to IFN-gamma modifies pulmonary inflammatory responses and abrogates immunity to Schistosoma mansoni in mice vaccinated with attenuated cercariae. J Immunol 149: 3654–3658.
45. Wilson RA, Coulson PS, Betts C, Dowling MA, Smythies LE (1996) Impaired immunity and altered pulmonary responses in mice with a disrupted interferon-gamma receptor gene exposed to the irradiated Schistosoma mansoni vaccine. Immunology 87: 275–282.
46. Sadler CH, Rutitzky LI, Stadecker MJ, Wilson RA (2003) IL-10 is crucial for the transition from acute to chronic disease state during infection of mice with Schistosoma mansoni. Eur J Immunol 33: 880–888.
47. Hogg KG, Kumkate S, Mountford AP (2003) IL-10 regulates early IL-12-mediated immune responses induced by the radiation-attenuated schistosome vaccine. Int Immunol 15: 1451–1459.

INFECTION AND IMMUNITY, June 2004, p. 3336–3343 Vol. 72, No. 6
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.6.3336–3343.2004

Recombinant *Mycobacterium bovis* BCG Expressing the Sm14 Antigen of *Schistosoma mansoni* Protects Mice from Cercarial Challenge

Paula B. Varaldo,[1] Luciana C. C. Leite,[1]* Waldely O. Dias,[1] Eliane N. Miyaji,[1] Fabio I. G. Torres,[1] Vera C. Gebara,[1] Geraldo R. G. Armôa,[2] Adriano S. Campos,[2] Denise C. S. Matos,[2] Nathalie Winter,[3] Brigitte Gicquel,[3,4] Mônica M. Vilar,[5] Johnjoe McFadden,[6] Marilia S. Almeida,[7] Miriam Tendler,[5]* and Douglas McIntosh[2]†

Centro de Biotecnologia, Instituto Butantan, São Paulo,[1] Laboratory of Recombinant Technology, Institute of Immunobiological Technology (Bio-Manguinhos),[2] and Department of Helminthology,[5] Oswaldo Cruz Foundation, Rio de Janeiro, and Microbiology and Parasitology Department, Federal University of Santa Catarina, Florianópolis,[7] Brazil; Laboratoire du BCG[3] and Unité de Génetique Mycobactérienne,[4] Institut Pasteur, Paris, France; and University of Surrey, Surrey, England[6]

Received 26 November 2003/Returned for modification 14 January 2004/Accepted 19 February 2004

The Sm14 antigen of *Schistosoma mansoni* was cloned and expressed in *Mycobacterium bovis* BCG as a fusion with the *Mycobacterium fortuitum* β-lactamase protein under the control of its promoter, pBlaF*; the protein was localized in the bacterial cell wall. The rBCG-Sm14 strain was shown to be relatively stable in cultured murine and bovine monocytes in terms of infectivity, bacterial persistence, and plasmid stability. The immunization of mice with rBCG-Sm14 showed no induction of anti-Sm14 antibodies; however, splenocytes of immunized mice released increased levels of gamma interferon upon stimulation with recombinant Sm14 (rSm14), indicating an induction of a Th1-predominant cellular response against Sm14. Mice immunized with one or two doses of rBCG-Sm14 and challenged with live *S. mansoni* cercaria showed a 48% reduction in worm burden, which was comparable to that obtained by immunization with three doses of rSm14 purified from *Escherichia coli*. The data presented here further enhance the status of Sm14 as a promising candidate antigen for the control of schistosomiasis and indicate that a one-dose regimen of rBCG-Sm14 could be considered a convenient means to overcome many of the practical problems associated with the successful implementation of a multiple-dose vaccine schedule in developing countries.

Schistosome infections, caused primarily by *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*, affect over 200 million people, mainly in the developing world, resulting in severe morbidity and mortality. In most cases the disease can be successfully treated by chemotherapy, but rapid reinfection rates for patients in endemic areas require repeated treatment, sometimes as often as once a year. Thus, there is a need for complementary long-term control measures, such as a vaccine (5, 6). Protection levels as high as 90% have been reported for the use of irradiated cercaria for immunization of a variety of animal models, ranging from rodents to nonhuman primates (11, 27). However, delivery problems, the need for a standardized product, and safety considerations combine to make this approach impractical for human use.

Extensive research on the biology of the parasite has identified six candidate antigens, which are currently included in a World Health Organization-sponsored program to develop a vaccine for the improved control of schistosomiasis (5, 6). Among these antigens is the fatty-acid-binding protein Sm14 of *S. mansoni* (23). This antigen, when produced as a recombinant protein by *Escherichia coli*, has been shown to form the basis for a dual-purpose antihelminth vaccine that is capable of conferring protection in outbred mice and rabbits against infection by *S. mansoni* and the ruminant helminth *Fasciola hepatica* (26, 30). The latter parasite is the causative agent of fascioliasis, an economically important disease of cattle and sheep in Europe, the Americas, and Australasia (12). The rationale for developing a schistosomiasis vaccine is multifactorial (6), but a key aspect is that a reduction in morbidity, rather than sterile immunity, is the target. Thus, a partially protective vaccine would be effective.

The bacillus Calmette-Guérin (BCG), a live attenuated *Mycobacterium bovis* strain that has been used for the prevention of human tuberculosis for decades, is considered a promising candidate for the development of live vector systems for the delivery of foreign antigens to the immune system (16, 25). Several advantages are associated with the use of BCG as an antigen-presenting system, including its known adjuvant properties, its ability to elicit humoral or cellular immune responses toward heterologous antigens, its thermostability, which eliminates the need for a cold chain, and most importantly, the possibility of obtaining an efficient immune response by using a single dose. During the last 10 years, expression systems have been developed for the production of a variety of bacterial, parasitic, and viral antigens by BCG, and the capacity of these recombinant systems to induce both cellular and humoral im-

* Corresponding author. Mailing address for Luciana Leite: Centro de Biotecnologia, Instituto Butantan, Av. Vital Brasil 1500, 05503-900 São Paulo SP, Brazil. Phone and fax: 55-11-3726-9150. E-mail: lccleite@butantan.gov.br. Mailing address for Miriam Tendler: Department of Helminthology, Instituto Oswaldo Cruz, Avenida Brasil 4365, CEP 21045-900 Rio de Janeiro, Brazil. Phone: 55-21-2598-4361. Fax: 55-21-2260-4866. E-mail: mtendler@ioc.fiocruz.br.

† Present address: Research and Productivity Council, Fredericton, Canada.

mune responses in various experimental models is well documented (2, 17, 20, 25). Furthermore, it has been demonstrated that recombinant BCG strains can induce protective immunity in animal models (1, 24, 29).

Recombinant BCG technology has previously been employed in the development of experimental schistosomiasis vaccines based on the enzyme glutathione *S*-transferase from *S. mansoni* (19) and *S. haematobium* (18). Both vaccines were found to elicit significant humoral immune responses toward the recombinant antigen when they were administered by a variety of routes, although no information is available about the potential protective capacities of these constructs.

For the present study, we developed a recombinant BCG strain which expresses the Sm14 antigen on the surface of the bacterium. Thereafter, the stability of the construct was evaluated in murine and bovine monocyte cultures. Immunization of mice with this strain induced an Sm14-specific Th1-based immune response, which paralleled a significant reduction in the worm burden in outbred Swiss mice challenged with *S. mansoni* cercaria.

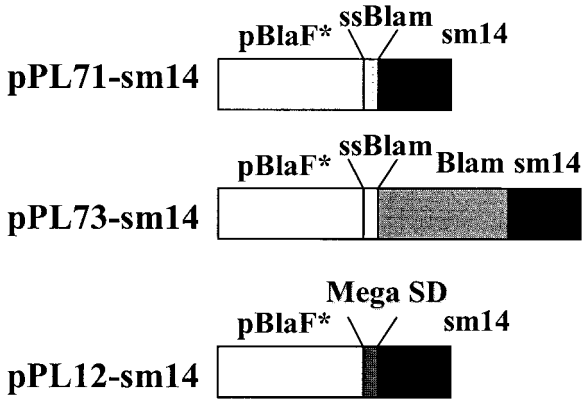


FIG. 1. Schematic representation of promoter and antigen regions of shuttle vectors pPL71-sm14, pPL73-sm14, and pPL12-sm14. All vectors contain *E. coli* and mycobacterial origins of replication, a kanamycin resistance gene (*aph*), pBlaF*, and *sm14*. pPL71-sm14 has the β-lactamase signal sequence (ssBlam) and pPL73-sm14 has the complete β-lactamase sequence (Blam) fused to the *sm14* sequence, while pPL12-sm14 has a conserved mycobacterial Shine-Dalgarno sequence (Mega SD).

MATERIALS AND METHODS

Bacterial strains, growth conditions, and vaccine preparation. All cloning steps were performed with *E. coli* DH5α grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) or kanamycin (20 μg/ml). The *M. bovis* BCG Pasteur strain 1172P2 was used to generate recombinant BCG (rBCG) strains. Liquid cultures of BCG strains were routinely grown in Middlebrook 7H9 medium supplemented with an albumin-dextrose-catalase enrichment (MB7H9; Difco, Detroit, Mich.), with or without kanamycin (20 μg/ml), in stationary 25-cm^2 tissue culture flasks at 37°C in a humidified 5% CO_2 atmosphere. The rBCG strains were cultured in Ungar's medium for heterologous protein localization assays. BCG was transformed by electroporation as previously described (25a) and plated onto Middlebrook 7H10 agar plates supplemented with an oleic acid-albumin-dextrose-catalase enrichment (MB7H10; Difco) containing kanamycin (20 μg/ml). Plates were incubated at 37°C for 3 weeks before expansion of the transformed colonies in liquid medium. rBCG vaccines were prepared from mid-log-phase liquid cultures of selected clones. The liquid cultures were centrifuged at 4,000 × *g*, resuspended in 10% glycerol, and maintained frozen at −80°C until use. Immediately before immunization, cells were thawed and diluted in saline to reach appropriate concentrations. Alternatively, for challenge assays, mice were immunized with fresh cultures of exponentially growing mycobacteria, with the actual vaccine dose calculated retrospectively by plating to determine viable numbers.

Plasmid vectors. pGEMEX-sm14 (containing the *sm14* gene (31), pGEM-T Easy (Promega), and the mycobacterial expression vectors pLA71, pLA73 (22), and pMIP12 (21) were used. The mycobacterial expression vectors contain the *E. coli* and mycobacterial origins of replication, a kanamycin resistance gene, the up-regulated *Mycobacterium fortuitum* promoter pBlaF*, its ATG initiation codon, and a multicloning site which places the heterologous gene in fusion with the signal sequence or the whole β-lactamase-encoding gene in pLA71 and pLA73, respectively. pMIP12 has a conserved mycobacterial Shine-Dalgarno sequence that may elevate antigen expression, and the native gene is expressed.

Construction of *sm14* expression vector. The 402-bp fragment containing the *sm14* gene was amplified by PCR from pGEMEX-sm14, using the following primers: 5′TAGGGTACCCTGTAGTTTCTTGGGAAAGTGGAA3′ (a KpnI site is underlined) and 5′TAGGGTACC*GCGGCCGC***GATATC**TTAGGATAG TCGTTTATAATTGC3′ (a KpnI site is underlined, an EcoRV site is in italics, and a NotI site is in bold) for insertion in pLA71 and pLA73 and 5′TAG*GGA TCC*TCTAGTTTCTTGGGAAAGTG3′ (a BamHI site is in italics) and 5′TAG GGTACCTTAGGATAGTCGTTTATAATTGC3′ (a KpnI site is underlined) for insertion in pMIP12. PCR fragments were cloned into pGEM-T Easy and then subcloned into the mycobacterial expression vectors, generating pPL71-sm14, pPL73-sm14, and pPL12-sm14, respectively (Fig. 1).

Western blotting and localization of heterologous proteins in rBCG. A Western blot analysis of individual kanamycin-resistant BCG transformants was performed by using a previously reported protocol (20), with the following modifications: sodium dodecyl sulfate-polyacrylamide gel electrophoresis employed 12% resolving gels and the presence of Sm14 was detected by use of a mouse polyclonal antiserum (1:1,000) raised against rSm14 (recombinant Sm14 purified from *E. coli*) as a primary antibody. Clones of rBCG expressing the heterologous protein (as determined by Western blotting) were grown in 30-ml cultures of Ungar's medium supplemented with kanamycin. Cells were harvested by centrifugation, and the culture supernatant and cell pellet were processed and analyzed as described previously (20).

In vitro stability of rBCG. Frozen aliquots of rBCG vaccine preparations were diluted (1/100 [vol/vol]) in MB7H9 medium and grown to an optical density at 594 nm of 0.8. A 1/100 dilution of this starting culture was further grown under the same conditions. Both cultures were used as inocula at suitable dilutions for plates of MB7H10 agar with or without kanamycin (20 μg/ml). The numbers of CFU recovered on kanamycin- versus non-kanamycin-containing plates were compared to estimate the functional plasmid stability.

Preparation of bovine and murine monocyte cultures. Blood (150 ml) from the jugular veins of two animals from a certified tuberculin-negative cattle herd held at the Rural University of Rio de Janeiro was collected into heparin-coated Vacutainers (Greiner). Blood was diluted in an equal volume of phosphate-buffered saline (PBS) (pH 7.2) and subjected to Ficoll gradient centrifugation. The coat containing white blood cells was collected, washed twice in PBS, and resuspended in culture medium (RPMI 1640, 2% fetal calf serum [FCS], 2 mM L-glutamine, 10 mM HEPES) (Gibco). Each suspension was divided between four tissue culture flasks and incubated for 7 days at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed after 3 days, and at day 7, the remaining nonadherent cells were removed by two washes with PBS, after which the adhered cells from each flask were gently scraped into 3 ml of PBS, pooled, and sedimented by centrifugation. Cells were resuspended in culture medium containing 10% FCS, plated on 24-well tissue culture plates (5×10^4 cells per well), and then incubated for a further 48 h prior to being used in infection experiments. Nearly confluent monolayers of the established murine macrophage cell line RAW 264.7 were produced from frozen stocks over a period of 4 days. The monolayers were washed once with PBS, scraped, and plated on 24-well plates (5×10^4 cells per well) 18 h prior to their use in infection experiments. The culture medium was the same as that used for the bovine cells, except for the FCS concentration, which was 1%.

Infection of monocyte cultures with BCG and rBCG. Cultures of BCG and rBCG (10 ml) were grown in tissue culture flasks to a density of ~10^8 cells/ml. For the production of homogeneous cell suspensions, cultures were transferred to centrifuge tubes, vortexed for 1 min, and then left standing for 15 min to allow the settlement of large clumps. The upper 8 ml of each suspension was carefully transferred to a new tube, and the bacteria were pelleted by centrifugation, washed twice in PBS, and finally resuspended in 10 ml of complete RPMI culture medium. Total cell counts were performed by direct counting, cell densities were adjusted to 10^6 bacteria/ml in complete RPMI medium, and 500 μl of the culture was added to each well containing bovine or murine cells, to achieve a theoretical multiplicity of infection (MOI) of 10 bacteria/macrophage. The actual number of

viable cells in each bacterial suspension was determined by preparing serial dilutions and plating them on MB7H10 agar. As such, the true MOI was determined retrospectively. In general, the number of viable cells was 20 to 50% lower than the value determined by microscopy, resulting in MOI values which ranged from 5 to 8 bacteria per macrophage, for four separate infection experiments. The bacteria were left in contact with the macrophages for 3 h, and then the monolayers were washed twice with 2 ml of PBS to remove the bulk of the nonphagocytosed bacteria. Thereafter, a sample comprising six wells from each plate of infected cells was processed to determine the infective capacity of rBCG compared to that of wild-type BCG. Briefly, monocytes/macrophages in six individual wells were lysed by incubation for 5 min in 0.2 ml of 0.05% Tween 80. The lysates were pooled, and the wells were then washed by the passage of a 0.8-ml volume of PBS from well to well, which was combined with the lysates. The lysate-washing mixtures were centrifuged at 10,000 × *g* for 10 min to sediment the released bacteria, the supernatants were removed by aspiration, the pellets were resuspended in 100 μl of MB7H9 medium and serially diluted to 10^{-4}, and triplicate 20-μl drops of each dilution were plated on MB7H10 agar, with or without kanamycin. Complete cell culture medium containing gentamicin (50 μg/ml) was added to the remaining 18 wells of each plate in order to kill extracellular bacteria that were not removed by washing. Sampling of the remaining infected cells was performed at 24 h and at 4 and 7 days as described above in order to provide data on the kinetics of persistence and the degree of plasmid maintenance by rBCG, as determined by a comparison of the bacterial numbers recovered on MB7H10 agar with or without kanamycin.

Evaluation of vector structural stability. Five individual colonies of rBCG-(pPL73-sm14), recovered from infected monocytes and sampled at 4 days postinfection, were used to transform *E. coli* DH5α by electroduction (4). Plasmid DNAs were purified from two individual transformants by use of a Flexiprep kit (Pharmacia) and were subsequently digested with the restriction endonucleases EcoRI and HindIII. Digested vectors were subjected to electrophoresis in 1% agarose gels, and the restriction pattern of this material was compared to that generated by digestion of the original vector with the same enzymes.

Animals and analysis of humoral immune response. Female 4-week-old BALB/c or Swiss mice (Instituto Butantan) were used for evaluations of the immune response against the rBCG strains. Mice were immunized intraperitoneally with 10^6 CFU/0.5 ml of BCG or an rBCG strain expressing Sm14. Positive and negative controls were three doses of 10 μg of rSm14 in alum per animal and three doses of saline, respectively. The animals were bled 4 weeks after the first dose. Animals received a booster dose 5 weeks after priming and a subimmunizing rSm14 dose (1 μg per animal) 7 weeks after priming. Antibody responses to Sm14 in pooled sera of immunized mice were quantified by enzyme-linked immunosorbent assays (ELISAs) using rSm14 (2 μg/ml in bicarbonate-carbonate buffer, pH 9.6, incubated at 4°C overnight) as the coating antigen. Thereafter, plates were processed as previously reported (24).

Preparation of spleen cells from immunized animals and lymphokine assays. Spleens were removed from two BALB/c or Swiss mice 4 weeks after immunization with rBCG(pPL73-Sm14) or the saline, BCG, and rSm14 controls. Splenocyte cultures were prepared as previously described (24) and incubated with rSm14 (1 μg/ml) as the antigen-specific stimulation and concanavalin A (5 μg/ml; Sigma) as a positive control for cell reactivity. Control cells were incubated in complete medium only, without antigen. Cultures were incubated for 3 days in a humidified 5% CO_2 incubator at 37°C. The culture supernatants were harvested at 24, 48, and 72 h in order to determine the optimal time for lymphokine secretion and were stored at −20°C until further quantification of gamma interferon (IFN-γ) and interleukin-4 (IL-4). IFN-γ and IL-4 levels in the supernatants of stimulated cells were quantified by ELISAs, as described elsewhere (24). Sample concentrations from standard curves were obtained by nonlinear regression analysis (Graph Pad Prism, version 2.0).

Challenge experiments. Two separate vaccine trials were established, with a gap of 1 month between their initiations. Each trial employed seven duplicate experimental groups (Table 1), with each group comprising 15 female 4-week old Swiss mice. The efficacy of vaccination with rBCG(pPL73-sm14) was compared to that achieved by the administration of three doses of rSm14 (10 μg in alum) in the foot pad. Liquid cultures of BCG and rBCG for use as vaccines were grown and evaluated for viability as described above, except that inocula were prepared in PBS rather than culture medium. Mice were immunized intraperitoneally with 10^6 CFU of BCG or rBCG(pPL73-sm14) in 0.1 ml of PBS, in single or duplicate doses. The viable number in each vaccine preparation was determined retrospectively as described above. For assays of protection, mice were challenged subcutaneously with 100 *S. mansoni* cercaria 60 days after the booster doses of BCG or rBCG and were perfused 45 days after the infective challenge. Since the results of the two trials were no more than 20% different, they were combined. Worm loads and protection levels for the different challenge groups were calculated as described previously (30). Statistical significance was evaluated by the standard Student *t* test.

TABLE 1. Imunization regimen employed to examine the capacity of rBCG-Sm14 to confer protection against challenge with *S. mansoni* cercaria

Vaccine trial	Content of immunization[a]	
	Primary (day 0)	Secondary (day 45)
Negative control	Saline in alum	None
BCG	BCG	None
	BCG	BCG
rBCG-Sm14	rBCG-Sm14	None
	rBCG-Sm14	rBCG-Sm14
	rBCG-Sm14	rSm14 (10 μg) in alum
Positive control	rSm14 (10 μg) in alum three doses 1 week apart)	

[a] The numbers of viable cells administered were as follows. Doses for trial 1: BCG primary dose, 8.4×10^5; secondary dose, 9.0×10^5; rBCG-Sm14 primary dose, 7.3×10^5; secondary dose, 8.5×10^5. Doses for trial 2: BCG primary dose, 6.3×10^5; secondary dose, 7.6×10^5; rBCG-Sm14 primary dose, 6.8×10^5; secondary dose, 1.0×10^6.

RESULTS

Expression and localization of recombinant Sm14 in BCG. Three mycobacterial expression vectors were constructed, based on the pBlaF* promoter, in which the *sm14* gene was or was not placed in fusion with different fragments of the β-lactamase gene (Fig. 1). Competent BCG Pasteur 1172P2 cells were transformed with these constructs, and the resulting recombinant clones were analyzed by immunoblotting using polyclonal anti-rSm14 antisera. All clones of rBCG(pPL73-sm14) were found to express a protein with a molecular mass of approximately 45 kDa, which corresponds to the expected molecular mass of the fusion product of the whole β-lactamase protein (31 kDa) and Sm14 (14 kDa) (Fig. 2a). The positive control containing 1 μg of purified rSm14 was seen at ~14 kDa. No degradation products were observed, indicating that the fusion protein was probably relatively stable. This protein was not detected in nontransformed BCG or BCG transformed with the empty vector, pLA73. Attempts to demonstrate the production of recombinant Sm14 by using chromogen-based detection (BCIP/NBT [5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium] in combination with alkaline phosphatase-labeled secondary antibodies) were unsuccessful, indicating that the level of expression was low. The use of constructs designed to express recombinant Sm14 in its native form (pPL12-sm14) or in fusion with only the β-lactamase signal sequence (pPL71-sm14) did not result in the recovery of any transformants expressing detectable levels of Sm14 (data not shown).

Cellular extracts of rBCG(pPL73-sm14) were fractionated by detergent phase partitioning with Triton X-114. A subsequent analysis of the subcellular fractions by Western blotting revealed that the Sm14 fusion protein was detected almost exclusively in the mycobacterial cell wall (Fig. 2b).

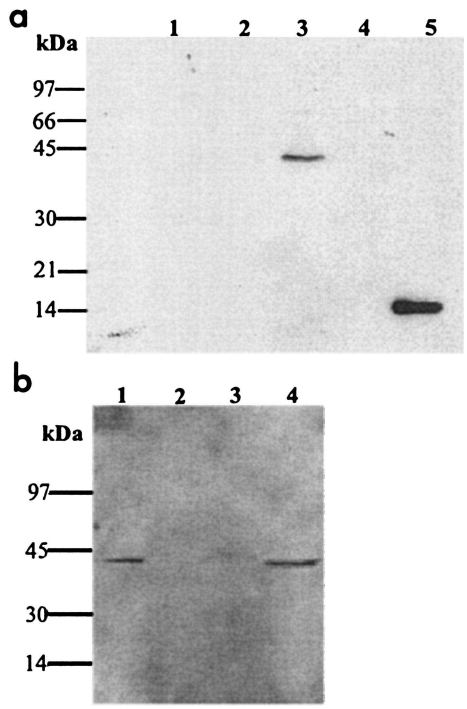


FIG. 2. Expression of Sm14 in rBCG. (a) Total cell extracts of BCG (50 μg) were analyzed by Western blotting using anti-rSm14 antiserum. Lanes: 1, BCG; 2, rBCG(pPL71-sm14); 3, rBCG(pPL73-sm14); 4, rBCG(pPL12-sm14); 5, control rSm14 (1 μg). (b) Localization of heterologous protein in rBCG(pPL73-sm14). Fractions of the extract of rBCG were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using anti-rSm14 antiserum. Lanes: 1, total extract; 2, membrane; 3, cytosol; 4, cell wall. Molecular masses are indicated to the left.

Behavior of rBCG(pPL73-sm14) in monocyte cultures. The infectivity, bacterial persistence, and plasmid stability of the rBCG(pPL73-sm14) strain were evaluated in both murine and bovine monocyte cultures. A total of four separate infection experiments were conducted, using adherent monocytes cultured from either the established murine cell line RAW 264.7 or two tuberculin-negative bovines. In terms of infectivity, we observed that between 5 and 10% of the bacterial inocula (BCG and rBCG) was taken up by the monocytes and recovered in the initial samples collected 3 h after contact with macrophages (Fig. 3). Regarding persistence, there was a gradual decline (as much as 2 log in one experiment, but more often a single log) in the numbers of BCG and rBCG recovered from RAW 264.7 cells over the 7-day experimental period (Fig. 3a). In the case of the bovine macrophage cultures, an increased decline in the numbers of BCG and rBCG was observed (routinely 2 log and exceptionally as much as 3 log) (Fig. 3b), indicating that these cells represent a more hostile environment than that presented by murine cells.

The issue of segregational vector stability (as measured in terms of loss of resistance to kanamycin) was assessed by comparing the number of CFU recovered on medium with and without kanamycin at each sampling point. We observed that at all points from 24 h onwards, approximately 10% of the cells recovered from both bovine and murine intracellular environments failed to grow on medium containing kanamycin (Fig. 3), suggesting that there had been a limited degree of plasmid loss. Interestingly, the pPL73-sm14 construct showed 100% stability for 7 days in the absence of selective pressure when grown in complete MB7H9 medium (data not shown), indicating that the observed plasmid loss in the macrophage cultures was in response to host factors which would likely exert a similar influence in vivo. In terms of structural vector stability, no gross modifications or rearrangements were observed for plasmids isolated from rBCG recovered after 4 days from macrophages of either species.

Immune response induced by rBCG expressing Sm14. In order to determine if rBCG(pPL73-sm14) could induce a humoral immune response against Sm14, we immunized BALB/c mice with rBCG-Sm14 or the positive and negative controls. A booster dose of rBCG-Sm14 and its negative controls was administered under the same conditions at 6 weeks. Sera were collected every 2 weeks and analyzed by ELISA for the formation of anti-Sm14 antibodies. No anti-Sm14 antibodies were detected in any of the groups, up to 4 weeks after the booster, except for the animals immunized with *E. coli*-derived rSm14

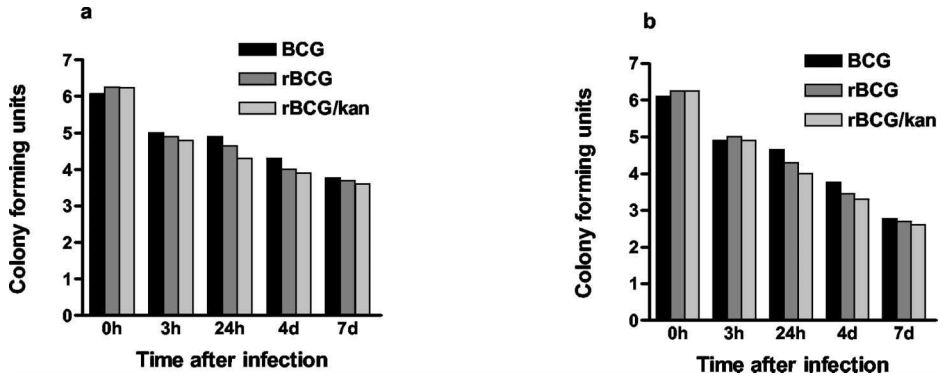


FIG. 3. Evaluation of infectivity, persistence, and plasmid maintenance of BCG and rBCG(pPL73-sm14) within murine (RAW 264.7) (a) and bovine (b) monocyte cultures. CFU are shown in $\log_{10}$ units. Time zero samples represent the total numbers of viable cells that were added to six wells (of a 24-well plate), i.e., the starting inocula. At each sample point, six wells were lysed and the number of CFU recovered on medium with and without kanamycin was recorded. Values recorded at 3 h represent the numbers of bacteria which had been internalized after 3 h of contact with the monocytes. As such, they provide a value for the infectivity of BCG and rBCG for each culture. The values recorded at 24 h and at 4 and 7 days give an indication of the persistence of each strain for the experimental period.

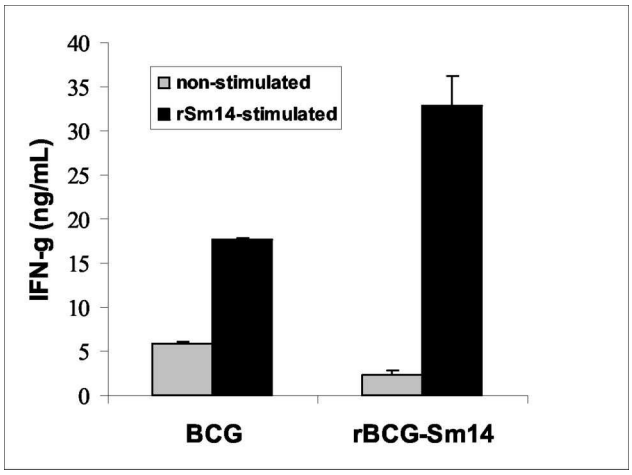


FIG. 4. Cellular response induced by immunization with rBCG-Sm14. The data show IFN-γ production by rSm14-stimulated splenocyte cultures from BALB/c mice immunized with BCG or rBCG-(pPL73-sm14) (10^6 CFU). The cytokine content was estimated as described in Materials and Methods. The error bars indicate standard deviations.

(results not shown). In order to determine if the rBCG-Sm14-immunized animals had been primed to respond to the antigen, a previously defined subimmunizing dose of rSm14 (1 μg/animal) was administered 2 weeks after the booster. However, even then, no anti-Sm14 antibodies were detectable after 2 and 4 weeks. These results indicate that rBCG-Sm14 does not induce a detectable humoral immune response against the antigen in BALB/c mice.

For an examination of cellular immune responses, BALB/c (or Swiss) mice were administered rBCG-Sm14, saline, or BCG (as described above). After 4 weeks, splenocytes were isolated for the quantification of Sm14-specific IFN-γ and IL-4 production. Splenocytes from mice immunized with rBCG-Sm14 consistently showed the most responsiveness to stimulation with rSm14, as shown in Fig. 4. As previously demonstrated, BCG-immunized animals also showed a nonspecific increase in IFN-γ production, although at consistently lower levels. IL-4 secretion in rBCG-Sm14-immunized mice was not significantly different from that in control groups (results not shown).

rBCG-Sm14 provides protection against cercarial infection. Groups of Swiss mice received one or two doses of rBCG-Sm14 (at an interval of 45 days). Another group received one dose of rBCG-Sm14 and one dose of rSm14, also after 45 days. Negative controls received one or two doses of BCG or three doses of saline, and the positive control group received three doses of rSm14. Sera were collected the day before each booster and 60 days after the last. Confirming the data obtained with BALB/c mice, neither one nor two doses of rBCG-Sm14 induced detectable levels of anti-Sm14 antibodies. However, one dose of rBCG-Sm14 followed by a full dose of rSm14 induced a low level of anti-Sm14 antibodies, with titer units of 1:320. This contrasts with the case seen for three doses of rSm14, with titer units reaching 1:2,560 but declining to a level of 1:640 before the challenge. Immunized animals were challenged subcutaneously with 100 live *S. mansoni* cercaria 60 days after the last immunization, and cercaria were recovered from the liver after 45 days by perfusion. Two trials were conducted 1 month apart, and the worm burden data were combined. The data presented in Fig. 5 and Table 2 demonstrate that a single dose of rBCG-Sm14 provided significant protection (~48%) for outbred Swiss mice, as measured by the reduction of the worm burden compared to that in nonvaccinated controls. The protection obtained was comparable to that achieved by using the standard three-dose schedule for rSm14 (Table 2). Interestingly, we observed that a booster dose of rBCG did not result in a significant enhancement of the protection obtained with a single dose (Table 2). Similarly, no improvement in protection was observed when a single booster dose of rSm14 was given 45 days after the primary immunization with rBCG-Sm14. Finally, the effect of either a single dose or duplicate doses of nontransformed BCG upon the worm

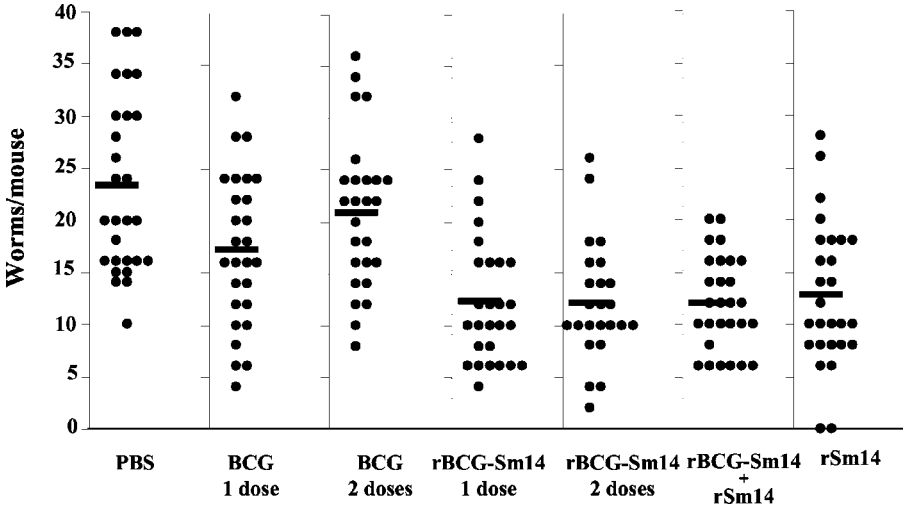


FIG. 5. Scattergram of worm burden in mice immunized with rBCG(pPL73-sm14) according to the immunization schedule shown in Table 1.

TABLE 2. Protection induced by rBCG-Sm14 against *S. mansoni* cercarial challenge

Immunization group[a]	Worm burden (no. of worms recovered after challenge with 100 worms)	Protection (%)	Significance (*P*)[b]
Saline	23.4		
Single dose of BCG	17.2	26	0.006
Two doses of BCG	20.9	11	0.25
Single dose of rBCG Sm14	12.2	48	1×10^{-6}
Two doses of rBCG Sm14	12.2	48	1×10^{-6}
Single dose of rBCG Sm14 boosted with single dose of rSm14 (10 μg in alum)	11.9	49	2×10^{-7}
Three doses of rSm14 (10 μg in alum)	12.7	46	5×10^{-6}

[a] Immunizations are described further in Table 1.
[b] By Student's *t* test.

burden was markedly lower (~26%) and most likely reflects a nonspecific adjuvant effect associated with BCG.

DISCUSSION

We have obtained expression of the *S. mansoni* antigen Sm14 in *M. bovis* BCG as a stable fusion with the whole *M. fortuitum* β-lactamase protein. It is noteworthy that vectors designed to express Sm14 that was not fused or was fused only with the β-lactamase signal sequence showed no detectable expression of the antigen. This observation mirrors the results of a previous study, in which the level of expression of the S1 subunit of pertussis toxin was markedly higher when this antigen was produced as a fusion with the entire β-lactamase protein rather than just the exportation peptide (24). This reinforces the hypothesis that some heterologous proteins need a transcription or translation start site from a mycobacterial protein in order to be expressed in BCG. On the other hand, a difference in codon usage could also hinder expression. Improved expression was a beneficial secondary effect of the fusion with the β-lactamase protein; however, the primary motive for employing this strategy was to obtain exportation of the antigen. Fractionation of the bacterium's cellular compartments into cytosol, membrane, cell wall, and extracellular media showed that the antigen was localized predominantly in the cell wall-enriched fraction. Once again these findings parallel previous results with the S1 subunit of pertussis toxin, for which neither a fusion with only the β-lactamase signal sequence nor a fusion with the whole β-lactamase protein resulted in complete exportation (24). We consider it unlikely that the size of the fusion protein (45 kDa) was responsible for the failure to attain secretion, given that BCG secretes a number of proteins with molecular masses of >45 kDa. Instead, our results further support the concept that size is not the only factor that influences the ability of such fusion molecules to translocate across the mycobacterial cell wall; additional characteristics of the heterologous protein should also play an important role.

In spite of promising results from numerous studies (reviewed in reference 25), no rBCG vaccine is currently licensed for use in either humans or animals. Indeed, only a single candidate rBCG vaccine, based on the expression of the OspA antigen from *Borrelia burgdorferi* (29), has been evaluated in a phase I human clinical trial (13). Surprisingly, this vaccine failed to induce immunity in humans, despite having being shown to be highly immunogenic and protective in various strains of mice (29). The reasons for this phenomenon remain unresolved, but they highlight the requirement for model systems with which to assess candidate rBCG vaccines in terms of their ability to infect, persist, and stably maintain their expression systems under conditions which resemble those encountered in the species for which the vaccine is being developed, or even as a preliminary step before embarking on costly small-animal experimentation. In this context, ex vivo macrophage cultures are commonly employed for the study of microbial (including mycobacterial) pathogenicity and host-pathogen interactions within intracellular environments (3, 28). Thus, we chose to evaluate the Sm14-expressing rBCG construct within macrophage cultures of both murine (our small-animal model) and bovine (an intended end user species, along with humans) origins, with the objective of detecting any major problems which might compromise its usefulness as a vaccine. Our results showed that the persistence of either BCG or rBCG declined with time in both murine, and more pronouncedly, bovine cells. Data from the study of Aldwell et al. (3) demonstrated that BCG Pasteur 1173P2 is capable of persistence, but not replication, in both bovine alveolar macrophages and murine peritoneal macrophages, although they did not observe the marked decrease in CFU recorded in the present study. It is possible that the stronger mycobactericidal activity observed for the bovine macrophages used in our study may have resulted from the cattle having been sensitized by natural exposure to environmental mycobacteria, although they had negative test results for tuberculin. This phenomenon has been suggested as a probable explanation for the highly variable efficacy of BCG vaccination in both humans (7) and cattle (10). In the bovine study, BCG vaccination was seen to be moderately effective in protecting cows against tuberculosis in two experimental trials but was ineffective in a third trial using cattle that were accredited as being tuberculosis-free. However, these cattle had high-level IFN-γ responses to purified protein derivative from *Mycobacterium avium*. The reduced capacity for BCG to persist in these animals compared to that in nonsensitized cattle was implicated as a factor in the failure of the vaccine.

The pPL73-sm14 construct transfected into BCG showed a high level of stability in the absence of selective pressure in vitro (100%) or in the hostile environment of mammalian macrophages (90%). The latter value is similar to the levels of segregational instability seen in mice inoculated with the related construct, pNL71-S1PT (86%) (24), an observation which supports the use of cultured macrophages as a model with which to investigate bacterial and plasmid stability in end-user species. The structural vector stability was also shown to be satisfactory, insofar as no gross modifications were detected in plasmids recovered from rBCG cultured from macrophage lysates. Taken together, these data provided a positive indication that the pPL73-sm14 construct was suitable for use in subsequent experiments with mice and should be able to stably maintain the expression vector if used as a vaccine for cattle.

In contrast to rSm14, which induced high levels of anti-Sm14 antibodies, rBCG-Sm14 induced little, if any, humoral immune response against Sm14. This was in agreement with the low levels of Sm-14-specific IL-4 secretion observed for splenocytes of immunized animals. Furthermore, the same splenocytes showed a marked increase in the production of IFN-γ upon stimulation with rSm14, indicating the induction of a strong Th1-driven cellular immune response. The marked differences noted for the two types of recombinant vaccines based on Sm14, in terms of the ability to stimulate humoral immunity, were not entirely unexpected given that they would be processed by and presented to the immune system by different mechanisms. In this context, it should be noted that the subcellular location of the recombinant antigen has been reported to influence both the quantity and quality of the immune response to a number of antigens produced by rBCG (15, 17, 29). Yet there are numerous examples of rBCG-based vaccines which are capable of inducing strong humoral immune responses to a variety of antigens (25). This reinforces the notion that the immune response induced against heterologous antigens expressed in rBCG is largely dependent on the antigen.

It is worth emphasizing that due to the well-known immune evasion mechanisms utilized by *S. mansoni*, the immune responses necessary for protection may differ from those generated during a natural infection, and therefore the importance of identifying immunological markers to anticipate vaccine efficacy should not be underestimated. In this context, the immunological basis for the protection against *S. mansoni* associated with rSm14 has been investigated in mice, but to date no concrete evidence has been presented to explain the mechanisms by which this molecule induces protection. An alternative approach for investigating this issue involved a characterization of the immune responses toward Sm14 in human populations living in areas of Brazil where schistosomiasis is endemic. A study conducted in an area of Minas Gerais State where schistosomiasis is endemic demonstrated that $CD4^+$ T cells from individuals considered to be naturally resistant to schistosomiasis produced IFN-γ and tumor necrosis factor alpha in response to rSm14 (8). Additionally, the anti-Sm14 serum antibody isotype profile of schistosomiasis patients predominantly comprised the immunoglobulin G1 (IgG1) and IgG3 subclasses, with only low levels of IgM, IgA, or IgE being present (9).

It is very interesting that both the induction of a strong humoral immune response against Sm14, such as that seen for the recombinant protein, and the induction of a predominantly cellular immune response, such as that observed for immunization with rBCG-Sm14, were able to reduce the worm burden and protect mice from a challenge with *S. mansoni* cercaria. However, it is worth considering that the absence of significant levels of IgG in response to rBCG-Sm14 may not truly reflect the role of the humoral immune response in the observed protection, since IgE and IgA isotypes have been suggested to have key roles in protection against schistosomal infections of humans in areas of endemicity, including Brazil (5). Future experiments with the rBCG-Sm14 strain should investigate this aspect in the mouse model.

In previous studies (26, 30), the rSm14 vaccine induced protection levels of up to 65% in outbred mice who received three doses of 10 μg each, and a level of 89% protection was recorded for rabbits immunized with three doses of 80 μg each (30). These results were highly encouraging, but it would be desirable to have a standardized recombinant vaccine which could achieve similar levels of protection when given as a single dose. Such a strategy would reduce the overall expense of any large-scale application of the vaccine and would counter the issue of noncompliance or the inability to adhere to a multidose vaccination schedule. In this context, the results of the present study are encouraging and further research on this topic would be worthwhile.

It is worth pointing out that BCG can be administered orally or intranasally. Interestingly, it has been reported (18) that the intranasal administration of an rBCG vaccine based on the expression of glutathione *S*-transferase from *S. haematobium* induces strong systemic and mucosal immune responses, including the production of anti-glutathione antibodies in the lung. It will be valuable to investigate this route of administration in future studies with the rBCG-Sm14 vaccine. This is particularly relevant since shortly after penetration of the skin of the mammalian host, the schistosomula migrate to the lungs and reside there for several days before they reach the liver, where they develop into sexually mature worms that then begin egg deposition. The Sm14 antigen is expressed from the somula to the adult phase, and effective anti-Sm14 immunity in the lungs could help to eliminate the infection at the earliest stage after parasitic invasion.

The data presented here further enhance the status of Sm14 as a promising candidate antigen for use in the control of schistosomiasis and show that the use of rBCG can be considered to represent a convenient means by which to overcome many of the practical problems associated with the successful implementation of a multiple dose vaccine schedule in developing countries.

ACKNOWLEDGMENTS

This research was supported by funding from the INCO-DEV (International Cooperation with Developing Countries) program of the European Community (project ICA4 CT-2000-30032). P.B.V. and E.N.M. were FAPESP fellows.

We are grateful to Maria Cristina Vidal Pessolani, Department of Tropical Medicine, Fiocruz, Brazil, for providing cultures of RAW 264.7 cells and to the staff of the Rural University of Rio de Janeiro, Brazil, for providing access to experimental animals and assistance in the collection of blood samples. We also thank Celso R. R. Ramos and Paulo Lee Ho (Instituto Butantan, Butantan, Brazil) for providing rSm14.

REFERENCES

1. **Abdelhak, S., H. Louzir, J. Timm, L. Blel, Z. Benlasfar, M. Lagranderie, M. Gheorghiu, K. Dellagi, and B. Gicquel.** 1995. Recombinant BCG expressing the leishmania surface antigen Gp63 induces protective immunity against Leishmania major infection in BALB/c mice. Microbiology **141:**1585–1592.
2. **Aldovini, A., and R. A. Young.** 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature **351:**479–482.
3. **Aldwell, F. E., D. N. Wedlock, L. J. Slobbe, J. F. Griffin, B. M. Buddle, and G. S. Buchan.** 2001. In vitro control of Mycobacterium bovis by macrophages. Tuberculosis (Edinburgh) **81:**115–123.
4. **Baulard, A., C. Jourdan, A. Mercenier, and C. Locht.** 1992. Rapid mycobacterial plasmid analysis by electroduction between Mycobacterium spp. and Escherichia coli. Nucleic Acids Res. **20:**4105.
5. **Bergquist, N. R.** 1998. Schistosomiasis vaccine development: progress and prospects. Mem. Inst. Oswaldo Cruz **93**(Suppl. 1)**:**95–101.
6. **Bergquist, N. R.** 2002. Schistosomiasis: from risk assessment to control. Trends Parasitol. **18:**309–314.
7. **Brandt, L., J. Feino Cunha, A. Weinreich Olsen, B. Chilima, P. Hirsch, R. Appelberg, and P. Andersen.** 2002. Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of

BCG and induction of protective immunity to tuberculosis. Infect. Immun. **70:**672–678.
8. **Brito, C. F., I. R. Caldas, P. Coura Filho, R. Correa-Oliveira, and S. C. Oliveira.** 2000. CD4+ T cells of schistosomiasis naturally resistant individuals living in an endemic area produce interferon-gamma and tumour necrosis factor-alpha in response to the recombinant 14kDa Schistosoma mansoni fatty acid-binding protein. Scand. J. Immunol. **51:**595–601.
9. **Brito, C. F., C. T. Fonseca, A. M. Goes, V. Azevedo, A. J. Simpson, and S. C. Oliveira.** 2000. Human IgG1 and IgG3 recognition of Schistosoma mansoni 14kDa fatty acid-binding recombinant protein. Parasite Immunol. **22:**41–48.
10. **Buddle, B. M., B. J. Wards, F. E. Aldwell, D. M. Collins, and G. W. de Lisle.** 2002. Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. Vaccine **20:**1126–1133.
11. **Coulson, P. S., and R. A. Wilson.** 1997. Recruitment of lymphocytes to the lung through vaccination enhances the immunity of mice exposed to irradiated schistosomes. Infect. Immun. **65:**42–48.
12. **Dalton, J. P., S. McGonigle, T. P. Rolph, and S. J. Andrews.** 1996. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. Infect. Immun. **64:**5066–5074.
13. **Edelman, R., K. Palmer, K. G. Russ, H. P. Secrest, J. A. Becker, S. A. Bodison, J. G. Perry, A. R. Sills, A. G. Barbour, C. J. Luke, M. S. Hanson, C. K. Stover, J. E. Burlein, G. P. Bansal, E. M. Connor, and S. Koenig.** 1999. Safety and immunogenicity of recombinant bacille Calmette-Guerin (rBCG) expressing Borrelia burgdorferi outer surface protein A (OspA) lipoprotein in adult volunteers: a candidate Lyme disease vaccine. Vaccine **17:**904–914.
14. **Gebara, V. C., V. L. Petricevich, I. Raw, and W. D. da Silva.** 1995. Effect of saponin from Quillaja saponaria (molina) on antibody, tumour necrosis factor and interferon-gamma production. Biotechnol. Appl. Biochem. **22:** 31–37.
15. **Grode, L., M. Kursar, J. Fensterle, S. H. Kaufmann, and J. Hess.** 2002. Cell-mediated immunity induced by recombinant Mycobacterium bovis bacille Calmette-Guerin strains against an intracellular bacterial pathogen: importance of antigen secretion or membrane-targeted antigen display as lipoprotein for vaccine efficacy. J. Immunol. **168:**1869–1876.
16. **Hanson, M. S., C. V. Lapcevich, and S. L. Haun.** 1995. Progress on development of the live BCG recombinant vaccine vehicle for combined vaccine delivery. Ann. N. Y. Acad. Sci. **754:**214–221.
17. **Himmelrich, H., R. Lo-Man, N. Winter, P. Guermonprez, C. Sedlik, M. Rojas, D. Monnaie, M. Gheorghiu, M. Lagranderie, M. Hofnung, B. Gicquel, J. M. Clement, and C. Leclerc.** 2000. Immune responses induced by recombinant BCG strains according to level of production of a foreign antigen: malE. Vaccine **18:**2636–2647.
18. **Kremer, L., L. Dupre, G. Riveau, A. Capron, and C. Locht.** 1998. Systemic and mucosal immune responses after intranasal administration of recombinant *Mycobacterium bovis* bacillus Calmette-Guerin expressing glutathione *S*-transferase from *Schistosoma haematobium*. Infect. Immun. **66:**5669–5676.
19. **Kremer, L., G. Riveau, A. Baulard, A. Capron, and C. Locht.** 1996. Neutralizing antibody responses elicited in mice immunized with recombinant bacillus Calmette-Guerin producing the Schistosoma mansoni glutathione *S*-transferase. J. Immunol. **156:**4309–4317.
20. **Lagranderie, M., R. Lo-Man, E. Deriaud, B. Gicquel, M. Gheorghiu, and C. Leclerc.** 1997. Genetic control of antibody responses induced by recombinant *Mycobacterium bovis* BCG expressing a foreign antigen. Infect. Immun. **65:**3057–3064.
21. **Le Dantec, C., N. Winter, B. Gicquel, V. Vincent, and M. Picardeau.** 2001. Genomic sequence and transcriptional analysis of a 23-kilobase mycobacterial linear plasmid: evidence for horizontal transfer and identification of plasmid maintenance systems. J. Bacteriol. **183:**2157–2164.
22. **Lim, E. M., J. Rauzier, J. Timm, G. Torrea, A. Murray, B. Gicquel, and D. Portnoi.** 1995. Identification of *Mycobacterium tuberculosis* DNA sequences encoding exported proteins by using *phoA* gene fusions. J. Bacteriol. **177:** 59–65.
23. **Moser, D., M. Tendler, G. Griffiths, and M. Q. Klinkert.** 1991. A 14-kDa Schistosoma mansoni polypeptide is homologous to a gene family of fatty acid binding proteins. J. Biol. Chem. **266:**8447–8454.
24. **Nascimento, I. P., W. O. Dias, R. P. Mazzantini, E. N. Miyaji, M. Gamberini, W. Quintilio, V. C. Gebara, D. F. Cardoso, P. L. Ho, I. Raw, N. Winter, B. Gicquel, R. Rappuoli, and L. C. Leite.** 2000. Recombinant *Mycobacterium bovis* BCG expressing pertussis toxin subunit S1 induces protection against an intracerebral challenge with live *Bordetella pertussis* in mice. Infect. Immun. **68:**4877–4883.
25. **Ohara, N., and T. Yamada.** 2001. Recombinant BCG vaccines. Vaccine **19:**4089–4098.
25a. **Parish, T., and N. G. Stoker.** 1995. Electroporation of mycobacteria. Methods Mol. Biol. **47:**237–252.
26. **Ramos, C. R., M. M. Vilar, A. L. Nascimento, P. L. Ho, N. Thaumaturgo, R. Edelenyi, M. Almeida, W. O. Dias, C. M. Diogo, and M. Tendler.** 2001. r-Sm14—pRSETA efficacy in experimental animals. Mem. Inst. Oswaldo Cruz **96**(Suppl. 1)**:**131–135.
27. **Richter, D., R. N. Incani, and D. A. Harn.** 1993. Isotype responses to candidate vaccine antigens in protective sera obtained from mice vaccinated with irradiated cercariae of *Schistosoma mansoni*. Infect. Immun. **61:**3003–3011.
28. **Stich, R. W., L. K. Shoda, M. Dreewes, B. Adler, T. W. Jungi, and W. C. Brown.** 1998. Stimulation of nitric oxide production in macrophages by *Babesia bovis*. Infect. Immun. **66:**4130–4136.
29. **Stover, C. K., G. P. Bansal, M. S. Hanson, J. E. Burlein, S. R. Palaszynski, J. F. Young, S. Koenig, D. B. Young, A. Sadziene, and A. G. Barbour.** 1993. Protective immunity elicited by recombinant bacille Calmette-Guerin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine. J. Exp. Med. **178:**197–209.
30. **Tendler, M., C. A. Brito, M. M. Vilar, N. Serra-Freire, C. M. Diogo, M. S. Almeida, A. C. Delbem, J. F. Da Silva, W. Savino, R. C. Garratt, N. Katz, and A. S. Simpson.** 1996. A Schistosoma mansoni fatty acid-binding protein, Sm14, is the potential basis of a dual-purpose anti-helminth vaccine. Proc. Natl. Acad. Sci. USA **93:**269–273.
31. **Tendler, M., M. M. Vilar, C. A. Brito, N. M. Freire, N. Katz, and A. Simpson.** 1995. Vaccination against schistosomiasis and fascioliasis with the new recombinant antigen Sm14: potential basis of a multi-valent anti-helminth vaccine? Mem. Inst. Oswaldo Cruz **90:**255–256.

Editor: D. L. Burns