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21/07/2006 10:19 DEMG

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Número (21)

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| DEPÓSITO Pedido de Patente ou de Certificado de Adição |  PI0603490 - 0 Espaço reservado para o recibo (número e data de depósito) | depósito / / |
|---|---|--------------|

Ao Instituto Nacional da Propriedade Industrial:

O requerente solicita a concessão de uma patente na natureza e nas condições abaixo indicadas:

1. Depositante (71):

- 1.1 Nome: UNIVERSIDADE FEDERAL DE MINAS GERAIS
- 1.2 Qualificação: ENSINO SUPERIOR 1.3 CGC/CPF: 17217985000104
- 1.4 Endereço completo: AV. ANTONIO CARLOS, 6627 - PAMPULHA - BELO HORIZONTE - MG
- 1.5 Telefone: 031 - 3499-4774
FAX: 031 - 3499-4027

 continua em folha anexa**2. Natureza:**

- 2.1 Invenção 2.1.1. Certificado de Adição 2.2 Modelo de Utilidade

Escreva, obrigatoriamente e por extenso, a Natureza desejada: PATENTE DE INVENÇÃO**3. Título da Invenção, do Modelo de Utilidade ou do Certificado de Adição (54):**
PROCESSO PARA VACINA RECOMBINANTE CONTRA... continua em folha anexa**4. Pedido de Divisão do pedido nº. , de .****5. Prioridade Interna - O depositante reivindica a seguinte prioridade:**

Nº de depósito Data de Depósito (66)

6. Prioridade - o depositante reivindica a(s) seguinte(s) prioridade(s):

| País ou organização de origem | Número do depósito | Data do depósito |
|-------------------------------|--------------------|------------------|
| | | |
| | | |
| | | |

 continua em folha anexa**7. Inventor (72):** *Ana Paula Salles Moura Fernandes*
 Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)
(art. 6º § 4º da LPI e item 1.1 do Ato Normativo nº 127/97)

- 7.1 Nome: ANA PAULA SALLÉS MOURA FERNANDES
- 7.2 Qualificação: PROFESSORA
- 7.3 Endereço: R. BERNARDO GUIMARÃES, 3101/302, BARRO PRETO, BELO HORIZONTE-MG
- 7.4 CEP: 30140083 7.5 Telefone 31-3499-4033

PT0003490

continua em folha anexa

8. Declaração na forma do item 3.2 do Ato Normativo nº 127/97:

em anexo

9. Declaração de divulgação anterior não prejudicial (Período de graça):
(art. 12 da LPI e item 2 do Ato Normativo nº 127/97):

em anexo

2

10. Procurador (74):

10.1 Nome

CPF/CGC:

10.2 Endereço:

10.3 CEP: 10.4 Telefone

11. Documentos anexados (assinale e indique também o número de folhas):
(Deverá ser indicado o nº total de somente uma das vias de cada documento)

| | | | |
|---|--------|---|---------|
| <input checked="" type="checkbox"/> 11.1 Guia de recolhimento | 1 fls. | <input checked="" type="checkbox"/> 11.5 Relatório descritivo | 20 fls. |
| <input type="checkbox"/> 11.2 Procuração | fls. | <input checked="" type="checkbox"/> 11.6 Reivindicações | 2 fls. |
| <input type="checkbox"/> 11.3 Documentos de prioridade | fls. | <input checked="" type="checkbox"/> 11.7 Desenhos | 13 fls. |
| <input type="checkbox"/> 11.4 Doc. de contrato de Trabalho | fls. | <input checked="" type="checkbox"/> 11.8 Resumo | 1 fls. |
| <input checked="" type="checkbox"/> 11.9 Outros (especificar): CNPJ, Diario Oficial, Declaração de Inventário | | | 6 fls. |
| 11.10 Total de folhas anexas: | | | 43 fls; |

12. Declaro, sob penas da Lei, que todas as informações acima prestadas são completas e verdadeiras

BH, 20/07/2006
Local e Data


Assinatura e Carimbo
Prof. Ronaldo Tadeu Pena

Rector

PI0603490

3

ANEXO 1 -

3. Título da invenção:

**"PROCESSO PARA VACINA RECOMBINANTE CONTRA A LEISHMANIOSE
VISCERAL CANINA CONTENDO O ANTÍGENO RECOMBINANTE A2 E QUE
PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE
ANIMAIS INFECTADOS".**

7. INVENTOR (72):

7.6 Nome: Christiane de Freitas Abrantes

7.7 Qualificação: Pesquisadora

7.8 Endereço: Rua Santa Catarina, n.º 908, ap 601, Bairro de Lourdes, Belo Horizonte - MG

7.9 CEP: 31270-080

7.10 Telefone: (31)3499-4033

7.11 Nome: Eduardo Antonio Ferraz Coelho

7.12 Qualificação: Pesquisador

7.13 Endereço: Rua Antonio Dias, n.º 707, ap 801, B. Santo Antonio, Belo Horizonte - MG

7.14 CEP: 30350-150

7.15 Telefone: (31)3499-4033

7.16 Nome: Ricardo Tostes Gazzinelli

7.17 Qualificação: Pesquisador

7.18 Endereço: R. Espírito Santo, n.º 2444, ap 701, Bairro de Lourdes, Belo Horizonte - MG

7.19 CEP: 30160-032

7.20 Telefone: (31)3499-4033

=

PI0603490

SIAFI2006-DOCUMENTO-CONSULTA-CONOB (CONSULTA ORDEM BANCARIA)

12/06/06 15:39 USUARIO : PALHARES
DATA EMISSAO : 12Jun06 TIPO OB: 10 NUMERO : 2006OB900219
UG/GESTAO EMITENTE: 153273 / 15229 - PRO-REITORIA DE PESQUISA/UFMG
BANCO : 001 AGENCIA : 1615 CONTA CORRENTE : 997380632
FAVORECIDO : 183038 / 18801 - INSTITUTO NACIONAL DA PROPRIEDADE INDUS
BANCO : 001 AGENCIA : 2234 CONTA CORRENTE : 997380632
DOCUMENTO ORIGEM : 153273/15229/2006RP000178
NUMERO BANCARIO : 001579410-5 PROCESSO :
INVERTE SALDO : NAO VALOR : 55,00

IDENT. TRANSFER. :

OBSERVACAO

DEPOSITO DE PEDIDO NACIONAL DE PATENTE DE INVENCÃO

| EVENTO INSCRICAO 1 | INSCRICAO 2 | CLASSIF.1 CLASSIF.2 | V A L O R |
|----------------------|-------------|---------------------|-----------|
| 531314 2006NE900001 | | 333913904 | 55,00 |
| 561602 0250159999400 | | | 55,00 |

LANCADO POR : 42226708634 - PALHARES UG : 153273 12Jun06 15:38.
PF1=AJUDA PF3=SAI PF4=ESPELHO PF12=RETORNA

LPI PROTOCOLO GERAL
nº do protocolo 014060008038
21/07/2006 10:19 DEMG



Gru Cobrança

Página 1 de 1

RECEIPO DO SACADO

BANCOTOBURSEN

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Local de Pagamento

Pagável em qualquer Banco

Vencimento

Cedente

INPI - Instituto Nacional da Propriedade Industrial

Agência/Código cedente

2234-9/333.028-1

Data do documento

06/2006

No documento

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Espécie doc.

RC

Acete

N

Data Proces.

12/06/2006

Uso do Banco

Carteira

18/027

Espécie

R\$

Quantidade

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Valor

Nosso Número

00.000.2.2.06.0306322.2

(=) Valor documento

R\$ 55,00

(-) Desconto / Abatimento

Processo:

Nosso Número Complementar:

RPI: Valor (R\$):

55,0

(-) Outras deduções

(+) Mora / Multa

(+) Outros acréscimos

(=)Valor Cobrado

R\$ 55,00

Governo Federal - Guia de Recolhimento da União. GRU - Cobrança

Sacado

Universidade Federal de Minas Gerais

Av. Antônio Carlos 6627 - Reitoria - 7º andar- sala 7005 Belo Horizonte MG, Belo Horizonte, BR/MG,
31270-901

Sacador/Avalista

Corte na linha pontilhada

Autenticação mecânica - Controle do Cedente



P 10603490

S

REPÚBLICA FEDERATIVA DO BRASIL
CADASTRO NACIONAL DA PESSOA JURÍDICA

NÚMERO DE INSCRIÇÃO
17.217.985/0001-04

COMPROVANTE DE INSCRIÇÃO E DE SITUAÇÃO
CADASTRAL

DATA DE ABERTURA
15/12/1966

NOME EMPRESARIAL
UNIVERSIDADE FEDERAL DE MINAS GERAIS

TÍTULO DO ESTABELECIMENTO (NOME DE FANTASIA)
REITORIA

CÓDIGO E DESCRIÇÃO DA ATIVIDADE ECONÔMICA PRINCIPAL
75.11-6-00 - Administração pública em geral

CÓDIGO E DESCRIÇÃO DA NATUREZA JURÍDICA
110-4 - AUTARQUIA FEDERAL

LOGRADOURO
AV ANTONIO CARLOS

NÚMERO
6627

COMPLEMENTO

CEP
31.270-901

BAIRRO/DISTRITO
PAMPULHA

MUNICÍPIO
BELO HORIZONTE

UF
MG

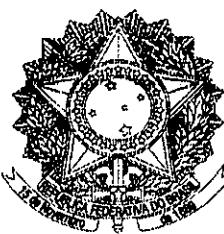
SITUAÇÃO CADASTRAL
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DATA DA SITUAÇÃO CADASTRAL
17/01/1998

SITUAÇÃO ESPECIAL

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CONFERE COM
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18/05/06
Sidcley L. Amoral Araújo
ASSISTENTE EN ADMINISTRAÇÃO REITORIA - CTAI
182370



DIÁRIO OFICIAL DA UNL

República Federativa do Brasil

Imprensa Naci

Ano XLVII N° 52

Brasília - DF, quinta-feira, 16 de março de 2006

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ATOS DO PODER EXECUTIVO

MINISTÉRIO DA EDUCAÇÃO

DECRETO DE 15 DE MARÇO DE 2006

O PRESIDENTE DA REPÚBLICA, no uso da atribuição que lhe confere o art. 84, inciso XXV, da Constituição, e tendo em vista o disposto no art. 16 da Lei nº 5.540, de 28 de novembro de 1968, resolve

NOMEAR

RONALDO TADEU PENA, Professor da Universidade Federal de Minas Gerais, para exercer o cargo de Reitor da referida Universidade, com mandato de quatro anos.

Brasília, 15 de março de 2006; 185º da Independência e 118º da República.

LUIZ INÁCIO LULA DA SILVA
Fernando Haddad

Presidência da República

SECRETARIA DE RELAÇÕES INSTITUCIONAIS

PORTARIA N° 31, DE 15 DE MARÇO DE 2006

O MINISTRO DE ESTADO CHEFE DA SECRETARIA DE RELAÇÕES INSTITUCIONAIS DA PRESIDÊNCIA DA REPÚBLICA, no uso da atribuição que lhe foi subdelegada pelo inciso I do art. 1º da Portaria nº 1.056, de 11 de junho de 2003, do Ministro de Estado Chefe da Casa Civil da Presidência da República, e tendo em vista o disposto no Decreto nº 5.526, de 26 de agosto de 2005, resolve:

NOMEAR

MANOEL MARIA COSTA QUINZEIRO FILHO, para exercer o cargo de Assistente Técnico, na Subchefia de Assuntos Federativos da Secretaria de Relações Institucionais da Presidência da República, código DAS 102.1.

JAQUES WAGNER

GABINETE DE SEGURANÇA INSTITUCIONAL

DESPACHO DO CHEFE

Processo nº (00181.000847/2006-15). Afastamento do País dos seguintes militares: 2º Sargento MARCOS ANTONIO GUEDES DA CRUZ, 3º Sargento JOSÉ LUIS MENDES e 3º Sargento LEONDINIS PEREIRA ROSA, no período de 13 a 15 de março de 2006, com ônus para a Presidência da República, a fim de compor a Equipe de Apoio da viagem à disposição da Presidência da República a cidade de Porto Príncipe - Haiti. Homologó. Em 15 de março de 2006.

Processo nº 00181.000802/2006-32. Afastamento do País do seguinte militar: Ten Cel Av IVAN MOYSÉS AYUPE, no período de 1º a 03 de março de 2006, com ônus, inclusive trânsito, por ter sido designado Piloto (Tripulante) da aeronave que resgatou, em Las Palmas (Espanha), os integrantes do Escalão Avançado composto pela equipe de apoio que precedeu a viagem Presidencial ao Reino Unido. Homologó. Em 15 de março de 2006.

JORGE ARMANDO FELIX

AGÊNCIA BRASILEIRA DE INTELIGÊNCIA

RETIFICAÇÃO

Na Portaria nº 169-ABIN/GSIPR, de 24/02/06, do Diretor-Geral da Agência Brasileira de Inteligência do Gabinete de Segurança Institucional da Presidência da República, publicada em extrato de acordo com o artigo 9º da Lei nº 9.883/99 e com o artigo 21 do anexo I ao Decreto 5.609/05, no Diário Oficial da União nº 43, de 03/03/06, página 2, Seção 2, que exonera servidor matrícula/ABIN nº 906791, a pedido, do cargo em comissão de que trata o § 2º do artigo 243 da Lei nº 8.112/90, onde se lê: "...matrícula/ABIN nº 906791, ...". leia-se "...matrícula/ABIN nº 907386, ...".

Na Portaria nº 189-ABIN/GSIPR, de 13/03/06, do Diretor-Geral da Agência Brasileira de Inteligência do Gabinete de Segurança Institucional da Presidência da República, publicada em extrato de acordo com o artigo 9º da Lei nº 9.883/99 e com o artigo 21 do anexo I ao Decreto 5.609/05, no Diário Oficial da União nº 50, de 14/03/06, página 2, Seção 2, que nomeia servidor matrícula/ABIN nº 908263, no cargo em comissão, código DAS 101.3, onde se lê: "...matrícula/ABIN nº 907386, ...".

Unidade Adm. III
UFMG / BIBLIOTECA UNIVERSITÁRIA
Av. Antônio Carlos, 6.627
Pampulha
BELO HORIZONTE
Diário Oficial da União - Seção 2
Distrib. Ricci Ltda - (031)3274-4136
Imprensa Pessoa
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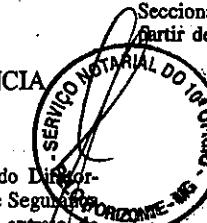
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| SERVIÇO NOTARIAL DO 10º OFÍCIO BELO HORIZONTE - MG | |
| Certifico que a presente cópia é idêntica ao original que me foi apresentado. Dto. de | |
| B.HORIZONTE MG | 27 ABR. 2006 |
| <input type="checkbox"/> Cláudio Alberto R. Araújo - Tab. Substituto <input type="checkbox"/> Cássia Muria de Souza - Tab. Substituta <input type="checkbox"/> Antônio Carlos Farid - Esc. Autorizado EMOL.: 2,30 F.C.R.: 0,14 F.J.: 0,77 TOTAL.: 3,21 | |



200603490

DECLARAÇÃO DO INVENTOR

Christiane de Freitas Abrantes, divorciada, brasileira, portadora da carteira de identidade n.º MG 10.155.034, expedida pelo SSP-MG, e do CPF n.º 019.716.474-97, residente e domiciliado na Rua Santa Catarina, n.º908, apto.601, Bairro de Lourdes, CEP 30170-080, Belo Horizonte/MG, doravante denominado Inventor, declara, para os devidos fins de direito, que:

tem conhecimento do requerimento de patente, cujo objeto é a tecnologia "Processo para vacina recombinante contra a leishmaniose visceral canina contendo o antígeno A2 e que permite a distinção sorológica entre animais vacinados e animais infectados", feito em nome da UFMG e dá pleno consentimento ao Instituto Nacional da Propriedade Industrial para conceder o privilégio.

Tendo em vista o acima exposto, o Inventor nada mais poderá reclamar em juízo ou fora dele qualquer direito referente à concessão do privilégio da patente da UFMG.

Este termo é assinado em condição irrevogável e irretratável.

Belo Horizonte, 12 de julho de 2006.



Christiane de Freitas Abrantes

Testemunhas:

1- Judson Fabrício Pinheiro

Nome:

CPF: 057.562.516-08

2- Sofia Dutra

Nome:

CPF: 058.826.906-92

PI0603490

DECLARAÇÃO DO INVENTOR

8

Eduardo Antonio Ferraz Coelho, solteiro, brasileiro, portador da carteira de identidade n.º M-6.526.944, expedida pelo SSP-MG, e do CPF n.º 893.510.886-34, residente e domiciliado na rua Antônio Dias, n.º 707, apto. 801, Bairro Santo Antônio, CEP. 30.350-150, Belo Horizonte/MG, doravante denominado Inventor, é pesquisador da Universidade Federal de Minas Gerais, declara, para os devidos fins de direito, que:

tem conhecimento do requerimento de patente, cujo objeto é a tecnologia “Processo para vacina recombinante contra a leishmaniose visceral canina contendo o antígeno A2 e que permite a distinção sorológica entre animais vacinados e animais infectados”, feito em nome da UFMG e dá pleno consentimento ao Instituto Nacional da Propriedade Industrial para conceder o privilégio.

Tendo em vista o acima exposto, o Inventor nada mais poderá reclamar em juízo ou fora dele qualquer direito referente à concessão do privilégio da patente da UFMG.

Este termo é assinado em condição irrevogável e irretratável.

Belo Horizonte, 13 de julho de 2006.

Eduardo Antonio Ferraz Coelho
Eduardo Antonio Ferraz Coelho

Testemunhas:

1- Anderson Fabrício Trindade

Nome:

CPF: 037.562.516-08

2- Sophia Dutra

Nome:

CPF: 058.826.906-92

P 00603490

DECLARAÇÃO DO INVENTOR

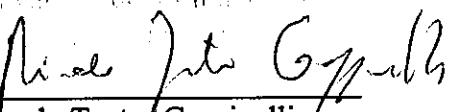
Ricardo Tostes Gazzinelli, casado, brasileiro, portador da carteira de identidade n.º M 1.147-255, expedida pela SSP/MG, e do CPF n.º 355.766.506-20, residente e domiciliado na Rua Espírito Santo, n.º 2444, apto. 701, Bairro Lourdes, CEP 30160-032, Belo Horizonte/MG, doravante denominado Inventor, é pesquisador da Universidade Federal de Minas Gerais, declara, para os devidos fins de direito, que:

tem conhecimento do requerimento de patente, cujo objeto é a tecnologia "Processo para vacina recombinante contra a leishmaniose visceral canina contendo o antígeno A2 e que permite a distinção sorológica entre animais vacinados e animais infectados, feito em nome da UFMG e dá pleno consentimento ao Instituto Nacional da Propriedade Industrial para conceder o privilégio.

Tendo em vista o acima exposto, o Inventor nada mais poderá reclamar em juízo ou fora dele qualquer direito referente à concessão do privilégio da patente da UFMG.

Este termo é assinado em condição irrevogável e irretratável.

Belo Horizonte, 12 de Julho de 2006.


Ricardo Tostes Gazzinelli

Testemunhas:

1- Anderson Ferreira Brandão

Nome:

CPF: 037.562.516-02

2- Sophia Dutra

Nome:

CPF: 058.226.906-92

Belo Horizonte, 12 de julho de 2006.

P1080340

DECLARAÇÃO DO INVENTOR

Ana Paula Salles Moura Fernandes, casada, brasileira, portador da carteira de identidade n.º m2717835, expedida pela SSP-MG, e do CPF n.º 62338757653, residente e domiciliado na Bernardo Guimarães, n.º 3101, apto. 302, Bairro Barro Preto, CEP30140-083, Belo Horizonte/MG, doravante denominado Inventor, declara, para os devidos fins de direito, que:

tem conhecimento do requerimento de patente, cujo objeto é a tecnologia “Processo para vacina recombinante contra a leishmaniose visceral canina contendo o antígeno A2 e que permite a distinção sorológica entre animais vacinados e animais infectados”, feito em nome da UFMG e dá pleno consentimento ao Instituto Nacional da Propriedade Industrial para conceder o privilégio.

Tendo em vista o acima exposto, o Inventor nada mais poderá reclamar em juízo ou fora dele qualquer direito referente à concessão do privilégio da patente da UFMG.

Este termo é assinado em condição irrevogável e irretratável.

Belo Horizonte, 13 de Julho de 2006.

Ana P. M. Fernandes
Ana Paula Salles Moura Fernandes

Testemunhas:

1- Luchan Fabrício Gundacker

Nome:

CPE: 937.562 . S16 - 08

2- Sophia Aita

Name:

CPF: 058.826.906-22

11

**PROCESSO PARA VACINA RECOMBINANTE CONTRA A LEISHMANIOSE
VISERAL CANINA CONTENDO O ANTÍGENO RECOMBINANTE A2 E QUE
PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE
ANIMAIS INFECTADOS.**

5 A presente invenção refere-se à vacina recombinante contra a leishmaniose visceral canina contendo a proteína recombinante A2 e saponina, como adjuvante, e que permite a distinção entre animais vacinados e infectados por meio de testes de ELISA ou imunofluorescência convencionais que empregam antígenos de formas promastigotas de *Leishmania*.

10 As leishmanioses constituem um grupo de doenças parasitárias que se apresentam clinicamente como lesões cutâneas, mucocutâneas ou sob a forma de infecção visceral e ocorrem devido à infecção por uma variedade de espécies de protozoários pertencentes ao gênero *Leishmania* (World Health Organization. Program for the surveillance and control of leishmaniasis.

15 <http://who.int/emc/diseases/leish/index.html>. 2005). As leishmanioses são endêmicas em cerca de 88 países. Destes, 72 são países em desenvolvimento e, neste grupo, estão incluídos 13 países com a menor taxa de desenvolvimento mundial. A forma visceral ocorre devido a infecções causadas pelas espécies *Leishmania* (*Leishmania*) *donovani* e *L. (L.) infantum* em países da Europa, Ásia, África e Oriente Médio e por *L. (L.) chagasi*, em países da América Latina (DESJEUX, P. Comp. *Immunol. Microbiol. Infect. Dis.*: 27, 2004). Alterações nas funções do baço, fígado e da medula óssea são observadas nos pacientes infectados, sendo que a infecção pode tornar-se crônica e causar febre irregular de longa duração, hepatoesplenomegalia, linfadenopatia, anemia, leucopenia, edema, debilidade progressiva e emagrecimento, podendo levar à morte na ausência de tratamento. Indivíduos infectados podem também permanecer assintomáticos, embora cerca de 20% dos indivíduos em regiões endêmicas desenvolvam a forma clássica da doença. Os sintomas são progressivos e as complicações decorrentes da evolução da infecção são responsáveis pela maioria dos óbitos (SUNDAR, S. & RAI, M. *Clin.Diagn. Lab. Immunol.*, 9: 2002).

20 A *L. (L.) chagasi* possui ampla distribuição geográfica nas Américas, sendo encontrada no Brasil, na Argentina, Colômbia, Bolívia, El Salvador, Guatemala, Honduras, México, Paraguai e Venezuela. Espécies como marsupiais e gambás são conhecidos reservatórios silvestres do parasita. Em ambientes domésticos, o cão é considerado o principal reservatório no ciclo de transmissão doméstico da leishmaniose visceral (LV) devido à elevada prevalência da infecção canina, quando

comparada à infecção humana. Cães infectados, mesmo assintomáticos, apresentam grande quantidade de parasitos na pele o que favorece a infecção do inseto vetor, a partir deste reservatório e, consequentemente, a transmissão para o homem (TESH, R. Am. J. Med. Hyg.: 52, 1995).

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5 O tratamento da LV canina, independente do fármaco utilizado, não é viável como medida de controle da doença, pois têm custo elevado e, freqüentemente, cães tratados e clinicamente curados sofrem recaídas, permanecendo como fontes de infecção para o vetor, além de aumentar o risco de seleção de linhagens resistentes a tais fármacos, com sérias implicações para o tratamento do homem (GRAMICCIA, M. & GRADONI, L. Int. J. Parasitol. 35: 2005).

10 Este fato, associado à letalidade da LV humana na ausência do tratamento, levou a Organização Mundial de Saúde (OMS) a preconizar a eliminação de cães quando soropositivos para antígenos de *Leishmania*, como medida de controle da infecção por órgãos de Saúde Pública. O Ministério da Saúde do Brasil, desta forma, 15 passou a adotar tal procedimento. Assim, uma das ações mais utilizadas no controle da LV é a eliminação dos cães infectados, os quais são detectados por meio do diagnóstico sorológico ou pela presença de sintomas clínicos. No entanto, tais procedimentos acarretam profunda tristeza e indignação aos seus donos que, por vezes, preferem omitir a doença aos órgãos competentes, até a proximidade da morte 20 dos animais quando, nesses casos, já se tornaram importantes transmissores do parasita (TESH, R. Am. J. Med. Hyg.: 52, 1995).

A maioria das pesquisas focando o desenvolvimento de vacinas baseia-se na identificação de moléculas do parasita e em protocolos de imunização que tenham a capacidade de induzir resposta imune celular Th1, requisito essencial para indução de 25 proteção à doença. Em cães, a resistência ou susceptibilidade à doença é também, provavelmente, associada à dicotomia da resposta Th1/Th2. A resistência é associada com a elevada resposta linfoproliferativa específica e com a reação de hipersensibilidade tardia (DTH) positiva, além de baixos títulos de anticorpos específicos ao parasita. A resistência à infecção e proteção, em cães, estaria 30 relacionada a uma elevada produção de interferon-gama (IFN- γ), de óxido nítrico (NO) e pela atividade leishmanicida dos macrófagos parasitados ou seja, a um perfil de resposta imune Th1. Níveis elevados de anticorpos IgG1 estariam associados à susceptibilidade, enquanto níveis elevados de IgG2a estariam associados à resistência (Moreno, J. & Alvar, J. Trends Parasitol.: 18, 2002; Molano, I. et al., Vet. 35 Immunol. Parasitol.: 92, 2003). Portanto, em estudos de avaliação de eficácia vacinal

contra a infecção por *Leishmania*, IFN-γ e anticorpos IgG2 (cães) ou IgG2a (camundongos) são utilizados como marcadores da resposta Th1 e de indução de resistência. Interleucina-4, interleucina-10 e anticorpos IgG1, ao contrário, são utilizados como marcadores de resposta Th2 e de susceptibilidade.

5 Vacinas contra Leishmaniose canina são de difícil desenvolvimento e, por isso, ainda raras. Uma vacina canina encontra-se disponível, a Leishmune®. Esta utiliza como ativo vacinal um complexo antigênico purificado, incluindo proteínas, que corresponde ao complexo Fucose-Manose Ligante (FML), presente na superfície do parasita, de acordo com o pedido de patente nacional no. PI 9302386-3 (Composição 10 contendo frações de células de leishmania, denominadas antígeno fml "fucose mannose ligand" ou "ligante de f ucose-manoze", uso do antígeno f ml e de suas subfrações e componentes para as aplicações em imunodiagnóstico específico da leishmaniose visceral humana e animal, para aplicações em vacinação, tratamento ou imunoterapia contra a leishmaniose visceral humana e canina).

15 A Leishmune® tem como característica primária a indução de resposta humoral. O cão vacinado desenvolve rapidamente resposta por meio da produção de anticorpos específicos contra o parasita. Diversos testes, apresentados pela inventora e parceiros da referida vacina, demonstram que a vacina protege cerca de 86% dos animais vacinados, quando inseridos em áreas endêmicas. Os dados destes estudos 20 foram questionados pela comunidade científica, bem como pela indústria, uma vez que os animais controle se localizavam em cidade distinta da que alocou os animais vacinados. Outra importante falha do teste citado foi a presença de cães, nos dois grupos, utilizando coleiras impregnadas com repelentes de insetos. O parasita é transmitido pela picada de um inseto, e se o contato entre inseto vetor é impedido pelo 25 uso de coleiras repelentes, o cão não está realmente exposto ao desafio natural preconizado. Com base nos dados acima é questionável o percentual de proteção descrito. Desta forma, outros estudos foram executados e alguns estão em execução, a pedido do órgão regulador em saúde pública.

Em particular, no que tange a saúde pública, é sabido que por 30 regulamentação da OMS, animais soropositivos devem ser sacrificados. Ainda, esta medida é adotada pelo Ministério da Saúde do Brasil. Uma vez vacinado com o produto em questão o animal desenvolverá pesada resposta por anticorpos específicos contra parasita, tornando-se soropositivo. O diagnóstico preconizado pelos órgãos públicos é o sorológico, por ser barato e de fácil execução podendo ser

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aplicado a todas as regiões do país sem maior ônus. Os cães vacinados deverão ser sacrificados.

A não diferenciação, por métodos tradicionais, de animais infectados daqueles vacinados apenas gera um grande problema para a saúde pública. Os donos de animais vacinados apresentam a carteira de vacinação e impedem que este seja sacrificado. Levando-se em conta que em cada cem animais vacinados cerca de 14 podem vir a se tornar infectados, causando o aumento de possíveis reservatórios domésticos para a leishmaniose. Outra questão importante está relacionada a inquéritos epidemiológicos realizados pelo Ministério da Saúde, que são importante forma de identificação da evolução da doença em diferentes regiões. Este inquérito se baseia, em parte, nos resultados sorológicos de cães. Com o advento da vacinação pela Leishmune® os resultados obtidos não são reais, pois animais soropositivos podem ter sido apenas vacinados e não infectados. Por meio de exames que detectam a presença do parasita, tais como a Reação em Cadeia da Polimerase (PCR) ou imunocitoquímica é possível diferenciar os cães soropositivos devido a vacinação ou infecção. No entanto, a execução destes exames exige técnicas aprimoradas, equipamento e reagentes de elevado custo, além de técnicos capacitados, para garantir fidelidade de resultados. A PCR é realizada em clínicas particulares, seu uso em saúde pública é de difícil implantação e de elevado custo financeiro.

Adicionalmente, a Leishmune® apresenta elevado custo de produção, chegando ao mercado a preços que impossibilitam o acesso de toda a população ao produto. O Brasil, sabidamente, possui uma larga faixa de sua população com baixa renda e este público não tem acesso ao produto citado. Sendo uma doença em expansão em diversas regiões do País, torna-se cada vez mais importante adotar medidas que impeçam a continuidade da transmissão do parasita, principalmente a infecção de cães, que habitam domicílios e peri domicílios. O possível uso deste produto em campanhas de saúde pública terá elevado custo, além dos problemas já citados. A Leishmune® tem características imunológicas interessantes, mas contrasta com as medidas de controle adotadas para a epidemia vigente impedindo o sacrifício de cães soropositivos, interferindo nos inquéritos epidemiológicos e ainda, sendo de elevado custo para uso na saúde pública.

O antígeno A2 foi identificado, inicialmente, na espécie *L. (L.) donovani*, por Charest & Matlashewski (*Mol. Cell. Biol.*: 14, 1994) a partir de uma biblioteca de cDNA de formas amastigotas de *L. (L.) donovani*. Cópias múltiplas do gene A2 estão

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agrupadas no cromossomo 22 (850 kb) de *L. (L.) donovani*, sendo que esses genes são conservados nas espécies *L. (L.) donovani*, *L. (L.) infantum*, *L. (L.) chagasi*, *L. (L.) amazonensis* e *L. (L.) mexicana* (Ghedin et al., *Clin. Diagn. Lab. Immunol.*: 4, 1997).

Em buscas conduzidas previamente em bancos de patentes, foram encontrados pedidos de patentes relacionados à utilização do antígeno A2 como reagente para vacinação contra a leishmaniose, conforme descrito a seguir. A patente de número US5733778 relata a seqüência de nucleotídeos do gene A2 e reivindica a proteção de sua expressão em hospedeiros microbianos. A seqüência do gene A2 de *L. (L.) donovani* cepa LV9, encontra-se depositada no Genebank, conforme descrito a seguir:

LOCUS S69693 2817 bp mRNA linear INV 26-MAR-2002

ACCESSION S69693

VERSION S69693.1 GI:546453

15 ORGANISM *Leishmania (donovani) infantum*

Eukaryota; Euglenozoa; Kinetoplastida; Trypanosomatidae;
Leishmania.

REMARK GenBank staff at the National Library of Medicine created this

FEATURES Location/Qualifiers

20 source 1..2817

/organism="Leishmania donovani infantum"

/mol_type="mRNA"

/strain="Ethiopian LV9"

/sub_species="infantum"

25 /db_xref="taxon:5662"

/dev_stage="amastigote"

gene 1..2817

/gene="A2"

CDS 72..782

30 /gene="A2"

/note="Plasmodium falciparum S antigen homolog; This sequence comes from Fig. 5A"

/codon_start=1

/product="stage-specific S antigen homolog"

35 /protein_id="AAB30592.1"

16

/db_xref="GI:546454"

ORIGIN

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 5 61 gaccgagcac aatgaagatc cgcagcgtgc gtccgttgt ggttgtctg gtgtcgctg
 121 cggcggtgct cgcaactcagc gcctccgctg agccgcacaa ggccggccgtt gacgtcgcc
 181 cgctctccgt tggccgcag tccgtggcc cgctctctgt tggccgcag gctgtggcc
 241 cgctctccgt tggccgcag tccgtggcc cgctctctgt tggccgcag gctgtggcc
 301 cgctctctgt tggccgcag tccgtggcc cgctctccgt tggccgcctc tccgtggcc
 10 361 cgcaagtctgt tggccgcctc tccgtggct cgcaagtccgt cggccgcctc tctgtggtc
 421 cgcaagtccgt cggccgcctc tccgtggcc cgcaaggctgt tggccgcctc tccgtggcc
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 541 cgcaagtccgt tggccgcctc tccgtggcc cgcaagtctgt tggccgcctc tccgtggct
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 721 cgcaagtccgt tggccgcctc tccgtggcc cgcaagtccgt tgacgttct ccggtgtctt
 781 aaggctccgc gtccgcttc cggtgtgcgt aaagtataatg ccataggca tggtgacgag
 841 gcaaaccttg tcagcaatgt ggcattatcg taccgtgtca agagcaacag cagagctgag
 901 tggcagggtg gccacagcac cacgtccgt tgacactccg tgggtgtgt gtgaccctgg
 20 961 ctgtgtgc caggcggatg aactgcgagg gccacagcac cgcaagtgcg gctccaacc
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 1081 agccatctag atgcgcctct ccacgacatg gccggaggcg gcagatgaag gcagcgaccc
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 1201 gtgcgcgggc gctgtgacgc acagccggca cgcaagctac cgacgcaga cagtcatgg
 25 1261 ggaggccgga ggagcaagag cgggtggacgg gaacggcgcg aagcatgcgg cacgcctcg
 1321 atgtgcctgt gtggctgtat gaggcgccga tgccgaaagc gtggcgaggg catcccgatg
 1381 tgcaccgtcg agtcctccag gcccgaatgt ggcgagcctg cggggaggcag attatggat
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 1501 tgcgcggcat ccgcctcgta tcgggagccc gaatggtggc cgcgccgt aaggcgtgcc
 1561 gcccacccgc gtcgttgtt ggccgcgtg gggcagggtg cgctgtggct gtgtatgtc
 1621 gctgtatgtc tgacttgttc gtgggtggct atgggcacgg tgagggcgaa cgttggccct
 1681 tgctgacttc ctgtgtttc ttattattct cagtcccccc gctggattgg gctgcacatgg
 1741 cgggtgtat cgccgttgtc tctctcattt gacggctgctg cgcctccgc ccctccact
 1801 cgtgtgtgg gatggaggca cggccggct ctgtgtgtg tgcacccgt gcaagaattc
 35 1861 agatgaggga ctgcccggcg agcagacaaa gcagcagcag caacaggaag gcaggccgtga

V

1921 gcacgtttc tttctctct tgagactgcg gactacggga atcagagacg tcgtcagaga
 1981 cgcgcatccg caccggcgcg ctatgttcc tcgttctctc tccggccccca ttctgtgcgc
 2041 ctgcctgtct gcgtgtcgcg agcgccgttg ccggcggctctctcccttcgttc
 2101 tctcttgc aa ggcgttcc ttcacagcc gaacgttgct gctgcctgg agggcgttcc
 5 2161 cccctttatc atctgtcat ttattttac acgtgtttt gcttggctt cctgacgtatc
 2221 ccggccacctt caccgggtt tcaggcccga ggcggccactc ttgtggca ggccaagtag
 2281 cctgcagccgtt gcccatgagc acggctgtgg actcttgggtt ccagcggaca ggtgtggct
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 2401 tgccgacgtatc tcaacgaatg cgcgcatttca cctactgcctt ttctgcctt gctgcgtgc
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 2521 gctgagcggc gccaggcggtt gctggccggc cctgctgtt ggcatacgcc tggcgtgcag
 2581 cagatcgaaa tgggtgtgg ctgcgtatgc gtgtgtgtt tgacttggc gtgggtggcg
 2641 ggcacgtaaa cggcaaaatg cgcttggcg ttccggcgcc acgctccggc gctggtgcgg
 2701 tattcaata cgcgcctgaa gaggtggcga gaaaaatggc acgaggcga gagggaaaaaa
 15 2761 acgaaaatgtt caaatgcgc aaaccgcga gaaaatgcgg gaaaaacgaa aagtgcga

Os pedidos de patente US570591, WO9506729, EP0716697 e MXPA03008832 relatam a seqüência de aminoácidos da proteína A2. A2 é composta por uma seqüência de dez aminoácidos, repetida de 40 a 90 vezes, dependendo do gene da "família A2" que a codifica (Charest & Matlashewski. *Mol. Cell. Biol.*: 14, 1994; Zhang et al., *Mol. Bioch. Parasitol.*:78, 1996), como indicado a seguir:

MKIRSVRPLVLLVCVAVLALSASAEPHKAAVDVGPLSVGPQSVGPLSVPQ
 25 AVGPLSVGPQSVGPLSVPQAVGPLSVGPQSVGPLSVPQSVGPLSVPGSQS
 VGPLSVGPQSVGPLSVPQAVGPLSVGPQSVGPLSVPQAVGPLSVGPQSVGPLSVP
 GPQSVGPLSVPGSQSVPQSVGPLSVPQSVGPLSVPQSVGPLSVPQSVGPLSVPQSV
 GPLSVPQSVDPVSPVS.

30 O pedido de patente US5780591, além de descrever a proteína nativa A2, ou seja, como ela é encontrada no parasita, reivindica a sua possível utilização como antígeno vacinal ou de diagnóstico. O pedido de patente US5733778 descreve a seqüência de DNA do gene A2 e sua expressão em bactérias. O pedido de patente US6133017 relata a obtenção de parasitas atenuados (*Leishmania donovani*) pela deleção do gene A2, bem como a sua utilização como vacinas atenuadas. Já as
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patentes WO9506729, EP0716697 ou MXPA03008832 são relacionadas entre si e reinvindicam a utilização do antígeno A2 sob a forma de proteína recombinante, DNA ou parasitas atenuados como vacina. No Brasil, foi registrado no INPI o pedido de patente PI0208532 (PCT número CA0200437), sob o título genérico de "vacina contra leishmania" e que relata a invenção de uma vacina de DNA cujo componente 5 antigenico é o antígeno A2, assim como dos processos para administrar esta vacina de DNA, a qual induz resposta imune no hospedeiro em que é administrada, contra a infecção por *Leishmania*.

As evidências experimentais que subsidiaram os pedidos de proteção das 10 vacinas acima, no entanto, consistem, essencialmente, em dois estudos de vacinação (Ghosh et al. *Vaccine*: 19, 2001; Ghosh et al., *Vaccine*: 20, 2002), em que modelos experimentais (camundongos) foram avaliados, empregando o antígeno A2, sob a proteína recombinante associada ao *Corynebacterium parvum*, como adjuvante, ou sob a forma de DNA com o plasmídeo pCDNA3/E6. De acordo com estes estudos, 15 animais imunizados com antígeno A2 e desafiados com *L. donovani* apresentaram redução significativa da carga parasitária no fígado, e uma produção elevada de IFN- γ e de anticorpos IgG2a específicos à proteína A2. Apesar da vacinação com DNA A2 ter conferido proteção, esta foi mais significativa quando este foi associado ao plasmídeo pCDNA3/E6. Embora necessária para a validação da eficácia de uma 20 formulação vacinal, a etapa de avaliação em modelos experimentais não pode ser considerada conclusiva mesmo que apresente resultados positivos.

O desenvolvimento de uma vacina que seja eficaz em proteger o cão e, consequentemente, diminuir as taxas de transmissão para o homem seria de grande relevância para o controle da leishmaniose. No Brasil, os Ministérios da Saúde e da 25 Agricultura, conforme portaria que se encontra em consulta pública (www.mapa.gov.br), preconizam que esta vacina, uma vez aplicada nos cães, seja capaz de induzir uma resposta imunológica eficaz em reduzir o parasitismo tecidual e a transmissão do parasita ao inseto vetor, o que pode ser evidenciado por meio de xenodiagnóstico, reação em cadeia da polimerase (PCR) ou imunohistoquímica. 30 Adicionalmente, esta vacina deve ser capaz de permitir a distinção sorológica dos cães vacinados daqueles infectados, empregando métodos laboratoriais de baixo custo, disponíveis na rede pública e que não onerem o sistema de saúde do País. Portanto, a adoção de uma vacina como nova medida de controle não deve interferir nas medidas de controle vigentes.

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A presente invenção propõe como solução para estes aspectos relacionados ao desenvolvimento de uma vacina aplicável ao contexto epidemiológico da leishmaniose, especialmente no Brasil, o emprego do antígeno amastigota específico A2 em preparações vacinais. Por se tratar de um antígeno específico do estágio amastigota de várias espécies de *Leishmania*, anticorpos gerados nos cães pelo processo de vacinação com este antígeno, não são reativos nos testes de diagnóstico sorológico da infecção, uma vez que na rotina laboratorial disponível na rede pública de saúde brasileira, estes testes utilizam抗igenos provenientes da forma promastigota do parasita, cuja obtenção tem custo mais baixo.

Após sucessivas avaliações e comprovação da eficácia do antígeno A2 em induzir proteção contra a infecção por *L. chagasi* e *L. amazonensis*, principais espécies de *Leishmania* que causam LV no Brasil, e de caracterizar a resposta imune induzida pela imunização com o antígeno A2 em camundongos, foi desenvolvida a formulação vacinal descrita a seguir, a ser aplicada em cães. Esta formulação foi então avaliada em cães quanto à capacidade de induzir resposta imune humoral e celular adequada ao seu emprego como vacina contra a LV no contexto epidemiológico desta doença em regiões em que o sacrifício de animais soropositivos é adotado como medida de controle. Atualmente, os testes de diagnóstico adotados na rede pública de saúde, não permitem a distinção sorológica entre animais infectados e aqueles que receberam vacinas constituídas de抗igenos provenientes de formas promastigotas do parasita. A formulação objeto do presente pedido, por ser constituída de um antígeno proveniente de formas amastigotas do parasita, possibilita tal distinção, evitando o sacrifício de animais saudáveis e que, portanto, não contraíram a infecção.

A invenção aqui descrita apresenta, portanto, uma nova formulação vacinal composta p ela p roteína r ecombinante A2 , as sociada à adj uvantes, a ex emplo de saponina, não limitante, e que apresenta comprovada eficácia em induzir proteção e resposta imune adequadas, não só em modelos experimentais, mas também em cães.

A formulação se constitui no antígeno A2 de *Leishmania*, produzido em *Escherichia coli*, sob a forma de proteína (ou antígeno) recombinante.

As fórmulas qualitativa e quantitativa apresentam:

Proteína recombinante A2-HIS (rA2)..... 50 a 200,00 µg/mL

Saponina..... 0,125 a 0,500 mg/mL

Solução salina tamponada ..q.s.p..... 1,00 mL

35 Thimerosol 0,01 mL

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Para a produção da proteína A2 recombinante, a seqüência codificante deste antígeno foi clonada no vetor de expressão de proteínas pET. A cepa BL21 de *Escherichia coli* foi transformada e, dessa forma, a proteína A2 foi expressa com cauda de 6 aminoácidos Histidina que permite a purificação da proteína recombinante por meio de cromatografia de afinidade com níquel. Testes de eletroforese em sistema de SDS-PAGE, de Western-Blot e o seqüenciamento de DNA confirmaram a identidade da proteína A2 clonada em *E. coli*. A expressão da proteína recombinante A2 (rA2) foi obtida após a indução da cultura de bactérias com 1,0 mM de IPTG (isopropil- β -D-tiogalactopiranósideo). A proteína rA2 foi purificada por cromatografia de afinidade em coluna contendo íons Níquel. Após a purificação, a integridade e pureza da proteína foram avaliadas por meio da técnica de Imuno Blot. O ajuste da concentração da proteína por mL é realizado com a utilização de solução s alina tamponada, contendo 0,5 mg de saponina e thimerosol.

A principal inovação desta formulação vacinal é a sua capacidade de induzir em cães resposta imune celular, caracterizada pela indução de níveis elevados de IFN- γ , e humoral, caracterizada pela produção de anticorpos específicos contra o antígeno vacinal, porém, que não reagem com o extrato bruto ou solúvel das formas promastigotas de *Leishmania* nos testes de ELISA ou na reação de imunofluorescência.

Desta forma, cães vacinados com esta formulação vacinal permanecem soronegativos após cada uma das doses de vacina necessárias ao processo de imunização sendo, portanto, possível a diferenciação sorológica entre os animais apenas vacinados com A2 daqueles infectados, os quais são soropositivos nos testes de ELISA, em reação com o extrato bruto ou solúvel dos parasitas.

Os resultados acima descritos podem ser demonstrados pelos seguintes exemplos:

Exemplo 1: níveis de proteção induzidos contra a infecção por *L. amazonensis* em camundongos BALB/c imunizados com o antígeno A2

A imunização com o antígeno A2, sob a forma da proteína recombinante A2 (rA2) associada à rIL-12, como adjuvante, ou sob a forma do DNA A2, foi eficaz em conferir proteção, em camundongos BALB/c, contra a infecção desafio com *L. (L.) amazonensis*. Na avaliação dos animais imunizados e desafiados, observou-se uma

redução significativa no tamanho médio das lesões e na carga parasitária nas patas infectadas (Figuras 1 e 2).

A Figura 1 apresenta a avaliação do tamanho médio das lesões nas patas infectadas de camundongos BALB/c imunizados com a proteína recombinante A2 (rA2), associada ou não à rIL-12, com rIL-12, DNA A2 ou DNA pCDNA3 e desafiados com *L. amazonensis*. Grupos de camundongos BALB/c (n=6, por grupo) foram imunizados com o DNA A2, no músculo tibial esquerdo, em intervalo de 21 dias. Grupos controle receberam apenas PBS ou foram imunizados com o plasmídeo vazio (DNA pCDNA3). Camundongos foram também imunizados, subcutaneamente, com a proteína rA2, em associação ou não à rIL-12 ou somente com rIL-12, como controle de adjuvante. Os camundongos foram desafiados com 1×10^6 promastigotas em fase estacionária de crescimento de *L. (L.) amazonensis*, no coxim plantar direito. A evolução do tamanho das lesões foi monitorada através das medidas semanais da espessura da pata infectada e da pata não infectada. Cada ponto representa a média acrescida ou subtraída do desvio padrão (em mm), obtida pela diferença de espessura entre a pata infectada e a pata não infectada.

A Figura 2 mostra a Avaliação da carga parasitária nas patas infectadas de camundongos BALB/c imunizados com a proteína recombinante A2 (rA2), associada ou não à rIL-12, com rIL-12, DNA A2 ou DNA pCDNA3 e desafiados com *L. amazonensis*. Grupos de camundongos BALB/c (n=6, por grupo) foram imunizados com 2 doses de 100 µg de DNA A2, no músculo tibial esquerdo, em intervalo de 21 dias. Grupos controle receberam apenas PBS ou foram imunizados com o plasmídeo vazio (DNA pCDNA3) (100 µg/dose). Camundongos foram também imunizados, subcutaneamente, com a proteína rA2, associada ou não à rIL-12 ou com rIL-12, como controle de adjuvante. 28 dias após a última dose, os camundongos foram desafiados com 1×10^6 promastigotas em fase estacionária de crescimento de *L. (L.) amazonensis*, no coxim plantar direito. Cerca de oito semanas após a infecção desafio, a carga parasitária nos animais (n=4, por grupo) foi determinada pela técnica de diluição limitante, conforme descrito na Seção de Material e Métodos. Cada barra representa a média acrescida ou subtraída do desvio padrão, referentes ao logaritmo do número de parasitas obtidos por miligrama de tecido. Diferenças entre as médias foram verificadas pelo teste t de Student, sendo consideradas significativas quando p inferior a 0,05.

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Exemplo 2: resposta imune celular e humoral em camundongos BALB/c imunizados com o antígeno A2 e desafiados com *L. amazonensis*

Animais imunizados com DNA A2 ou com rA2/rIL-12 e desafiados com *L. (L.) amazonensis* apresentaram uma produção significativa de IFN- γ , após o estímulo *in vitro* dos esplenócitos com a proteína rA2 ou com o extrato total de promastigotas (SLA) de *L. (L.) amazonensis* (Figura 3). Adicionalmente, baixos níveis de IL-4 e IL-10 foram observados nestes grupos, em comparação aos grupos controle, após o estímulo das células com a proteína rA2 ou com o SLA de *L. (L.) amazonensis* (Figura 4 e Figura 5).

A Figura 3 é um gráfico que representa a produção de IFN- γ por esplenócitos de camundongos BALB/c imunizados com a proteína recombinante A2 (rA2), associada ou não à rIL-12, com rIL-12, DNA A2 ou DNA pCDNA3 e desafiados com *L. (L.) amazonensis*. Células do baço de camundongos BALB/c foram coletadas antes da infecção desafio ou cerca de 8 a 9 semanas após o desafio com imunizados com *L. (L.) amazonensis*. As células foram cultivadas (1×10^6 /mL) em 1 mL de meio DMEM completo e estimuladas com a proteína rA2 (10 μ g/mL) e com o SLA de *L. (L.) amazonensis* (50 μ g/mL). Como controles, foram avaliadas as células não estimuladas ou estimuladas com concanavalina A (5 μ g/mL), para avaliar a viabilidade celular. Os cultivos foram incubados por 48 horas em estufa a 37°C, com presença de 5% de CO₂. Após, o sobrenadante foi coletado e a produção de IFN- γ foi determinada por ELISA de captura. No eixo das abscissas, encontra-se indicado o estímulo utilizado no cultivo celular, além do momento em que as células foram coletadas: antes ou após a infecção desafio. No eixo das ordenadas, a concentração, em pg/mL, de IFN- γ . Cada barra representa a média da produção de IFN- γ acrescido ou subtraído do desvio padrão de cada grupo.

Na Figura 4 está representada a produção de IL-4 por esplenócitos de camundongos BALB/c imunizados com a proteína recombinante A2 (rA2), associada ou não à rIL-12, com rIL-12, DNA A2 ou DNA pCDNA3 e desafiados com *L. (L.) amazonensis*. Células do baço de camundongos BALB/c foram coletadas antes da infecção desafio ou cerca de 8 a 9 semanas após o desafio com imunizados com *L. (L.) amazonensis*. As células foram cultivadas (1×10^6 /mL) em 1 mL de meio DMEM completo e estimuladas com a proteína rA2 (10 μ g/mL) e com o SLA de *L. (L.) amazonensis* (50 μ g/mL). Como controles, foram avaliadas as células não estimuladas ou estimuladas com concanavalina A (5 μ g/mL), para avaliar a viabilidade celular. Os

cultivos foram incubados por 48 horas em estufa a 37°C, com presença de 5% de CO₂. Após, o sobrenadante foi coletado e a produção de IL-4 foi determinada por ELISA de captura. No eixo das abscissas, encontra-se indicado o estímulo utilizado no cultivo celular, além do momento em que as células foram coletadas: antes ou após a infecção desafio. No eixo das ordenadas, a concentração, em pg/mL, de IL-4. Cada barra representa a média da produção de IL-4 e o desvio padrão de cada grupo.

Na Figura 5 encontra-se o gráfico demonstrativo da produção de IL-10 por esplenócitos de camundongos BALB/c imunizados com a proteína recombinante A2 (rA2), associada ou não à rIL-12, com rIL-12, DNA A2 ou DNA pCDNA3 e desafiados com *L. amazonensis*. Células do baço de camundongos BALB/c foram coletadas antes da infecção desafio ou cerca de 8 a 9 semanas após o desafio com imunizados com *L. amazonensis*. As células foram cultivadas (1×10^6 /mL) em 1 mL de meio DMEM completo e estimuladas com a proteína rA2 (10 µg/mL) e com o SLA de *L. amazonensis* (50 µg/mL). Como controles, foram avaliadas as células não estimuladas ou estimuladas com concanavalina A (5 µg/mL), para avaliar a viabilidade celular. Os cultivos foram incubados por 48 horas em estufa a 37°C, com presença de 5% de CO₂. Após, o sobrenadante foi coletado e a produção de IL-10 foi determinada por ELISA de captura. No eixo das abscissas, encontra-se indicado o estímulo utilizado no cultivo celular, além do momento em que as células foram coletadas: antes ou após a infecção desafio. No eixo das ordenadas, a concentração, em pg/mL, de IL-10. Cada barra representa a média da produção de IL-10 acrescido ou subtraído do desvio padrão de cada grupo.

Na avaliação da resposta imune humoral (Figura 6), amostras de soro coletadas dos animais imunizados com rA2/rIL-12 ou com DNA A2 e desafiados com *L. amazonensis* mostraram uma produção elevada de anticorpos IgG2a específicos à proteína rA2 (anti-rA2) e uma baixa produção de anticorpos específicos ao parasita (anti-SLA), contrariamente ao observado nos grupos controle (COELHO et al. *Infect. Immun.*: 71, 2003; COELHO. TESE, Doutorado em Imunologia. Belo Horizonte: Instituto de Ciências Biológicas da UFMG, 2004)

A Figura 6 mostra a produção de IgG total em amostras de soro de camundongos BALB/c imunizados com a proteína recombinante A2 (rA2), associada ou não à rIL-12, com rIL-12, DNA A2 ou DNA pCDNA3 e desafiados com *L. amazonensis*. Amostras de soro de camundongos BALB/c foram coletadas antes ou 8 a 9 semanas após a infecção desafio com *L. amazonensis*. As placas foram sensibilizadas com a proteína rA2 (250 ng/well) ou com o SLA de *L. amazonensis*. A produção de IgG total foi

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determinada por ELISA direta. No eixo das abscissas encontra-se a indicação da classe IgG total específica à proteína rA2 ou ao SLA de *L. amazonensis*, além do momento em que as amostras foram coletadas: antes ou após a infecção desafio. No eixo das ordenadas, a absorbância em comprimento de onda de 492 nm. Cada barra 5 representa a média da produção de IgG total adicionado ou subtraído o desvio padrão de cada grupo.

Na avaliação da produção das sub-classes IgG1 e IgG2a específicos à proteína rA2 ou a抗igenos do parasita (SLA), observa-se (Figura 7), que amostras de soro coletadas dos animais imunizados com rA2/rIL-12 ou com DNA A2 e desafiados com *L. amazonensis* mostraram um predomínio na produção de anticorpos IgG2a específicos à proteína rA2 (anti-rA2), em relação aos níveis obtidos de anticorpos IgG1 anti-rA2. Animais imunizados com A2 permaneceram, no entanto, soronegativos em testes 10 utilizando-se抗igenos de formas promastigotas de *Leishmania* (SLA) (Coelho et al., 2003; Coelho, 2004).

A Figura 7 descreve a produção de IgG1 e IgG2a em amostras de soro de camundongos BALB/c imunizados com a proteína recombinante A2 (rA2), associada ou não a rIL-12, com rIL-12, DNA A2 ou DNA pCDNA3 e desafiados com *L. amazonensis*. Amostras de soro de camundongos BALB/c foram coletadas antes ou 8 a 9 semanas após a infecção desafio com *L. amazonensis*. As placas foram 15 sensibilizadas com a proteína rA2 (250 ng/well) ou com o SLA de *L. amazonensis*. A produção de IgG1 e IgG2a foi determinada por ELISA indireta. No eixo das abscissas encontra-se a indicação das subclasses IgG1 ou IgG2a, específicas à proteína rA2 ou ao SLA de *L. amazonensis*, além do momento em que as amostras foram coletadas: antes ou após a infecção desafio. No eixo das ordenadas, a absorbância em 20 comprimento de onda de 492 nm. Cada barra representa a média da produção de IgG1 ou IgG2a e o desvio padrão de cada grupo.

Exemplo 3: níveis de proteção induzidos contra a infecção por *L. chagasi* em camundongos BALB/c imunizados com o antígeno A2

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O antígeno A2, quando administrado sob a forma de DNA A2, mostrou-se também eficaz em conferir proteção em camundongos BALB/c, contra a infecção desafio com *L. chagasi*, o principal agente etiológico da Leishmaniose Visceral nos países da América do Sul. Os animais imunizados e desafiados apresentaram uma 35 redução expressiva na carga parasitária no fígado (Figura 8) e no baço (Figura 9).

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Figura 8: Avaliação da carga parasitária no fígado de camundongos BALB/c imunizados com o DNA A2 e desafiados com *L. chagasi*. Grupos de camundongos BALB/c (n=6, por grupo), após os protocolos de imunização, foram desafiados com 1×10^7 promastigotas em fase estacionária de crescimento de *L. chagasi*, por via endovenosa, conforme descrito na Seção de Material e Métodos. 35 dias após o desafio, os animais (n=4, por grupo) foram sacrificados e o fígado foi recuperado, para a determinação da carga parasitária. As barras representam a média do log do número de parasitas por órgão acrescido ou subtraído do desvio padrão de cada grupo. A média do log do número total de parasitas por órgão foi comparada através do teste t de Student. Diferenças estatísticas foram consideradas significativas quando p é menor do que 0,05. Houve redução significativa na carga parasitária hepática dos animais do grupo DNA A2 em relação aos grupos PBS (p é menor do que 0,0005) e DNA pCI (p é menor do que 0,005).

A Figura 9 é o resultado da avaliação da carga parasitária no baço de camundongos BALB/c imunizados com o DNA A2 e desafiados com *L. chagasi*. Grupos de camundongos BALB/c (n=6, por grupo), após os protocolos de imunização, foram desafiados com 1×10^7 promastigotas em fase estacionária de crescimento de *L. chagasi*, por via endovenosa. 35 dias após o desafio, os animais (n=4, por grupo) foram sacrificados e o baço foi recuperado, para a determinação da carga parasitária. As barras representam a média do log do número de parasitas por órgão acrescido ou subtraído do desvio padrão de cada grupo. A média do log do número total de parasitas por órgão foi comparada através do teste t de Student. Diferenças estatísticas foram consideradas significativas quando p é menor do que 0,05. Houve redução significativa na carga parasitária esplênica nos animais do grupo DNA A2, em relação aos animais dos grupos PBS e DNA pCI (p é menor do que 0,005 e p é menor do que 0,05, respectivamente).

Exemplo 4: resposta imune celular em camundongos BALB/c imunizados com o antígeno A2 e desafiados com *L. amazonensis*

Camundongos BALB/c imunizados com o antígeno A2, sob a forma de DNA, e desafiados com *L. chagasi* produziram níveis significativamente maiores de IFN- γ (Figura 10) e menores de IL-10 (Figura 11), após o estímulo dos esplenócitos com a proteína rA2 ou com o extrato total de proteínas (LcPA) de *L. chagasi*, em relação aos animais dos grupos controle.

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Figura 10: gráfico da produção de IFN- γ por esplenócitos de camundongos BALB/c imunizados com DNA A2 antes e após desafio com *L. chagasi*. Células esplênicas de camundongos BALB/c imunizados foram coletadas 4 semanas após a imunização ou cerca de 9 semanas após a infecção desafio com *L. chagasi*. As células (2x10⁵ células por mL) foram cultivadas em DMEM completo e estimuladas com as proteínas recombinantes rNH, rA2 ou rLACK (concentrações de 10 µg/mL) ou com o extrato de proteínas de *L. chagasi* (50 µg/mL). Os sobrenadantes foram coletados 48 horas após o cultivo e a estimulação das células. A produção de IFN- γ foi determinada por ELISA de captura. No eixo das abscissas, estão indicados os estímulos utilizados no cultivo celular e no eixo das ordenadas a concentração de IFN- γ (pg/mL). Cada barra representa a média acrescida ou subtraída do erro padrão da produção de IFN- γ de cada grupo. A produção de IFN- γ basal corresponde a 428,0 acrescido ou subtraído de 66,45 pg/mL. (*) indica diferença significativa em relação aos grupos PBS e DNA controle, avaliados pelo teste t de Student, considerados significativos os valores em que p é menor do que 0,05.

A figura 11 demonstra Produção de IL-10 por esplenócitos de camundongos BALB/c imunizados com plasmídeos de DNA NH, DNA A2, DNA LACK ou de suas associações, antes e após o desafio com *L. chagasi*. Grupos de camundongos BALB/c (n=6, por grupo), após os protocolos de imunização, foram desafiados com 1x10⁷ promastigotas em fase estacionária de crescimento de *L. chagasi*, por via endovenosa, conforme descrito na Seção de Material e Métodos. 28 dias após a imunização ou 35 dias após a infecção desafio, as células esplênicas foram cultivadas em meio DMEM completo e estimuladas com as proteínas recombinantes rNH, rA2 ou rLACK (10 µg/mL) ou com o extrato solúvel de *L. chagasi* (LcPA, 50 µg/mL). Os sobrenadantes foram coletados 48 horas após o cultivo e a produção de IL-10 foi determinada por ELISA de captura. No eixo das abscissas, estão indicados os estímulos utilizados no cultivo celular e, no eixo das ordenadas, a concentração de IL-10 (pg/mL). Cada barra representa a média da produção de IL-10 adicionado ou subtraído o erro padrão de cada grupo. A produção de IL-10 basal corresponde a 653,5 acrescido ou subtraído de 59,6 pg/mL. (*) indica diferença significativa em relação ao grupo PBS e (**) indica diferença significativa em relação aos grupos PBS e DNA pCI. Análise estatística pelo teste t de Student, sendo as diferenças consideradas significativas quando p é menor do que 0,05.

Exemplo 5: resposta imune celular e humoral induzida pela imunização com o antígeno A2 em cães da raça Beagle

Cães da raça Beagle foram distribuídos em grupos e imunizados com três doses da formulação vacinal antígeno A2/saponina, em intervalos de 21 dias, pela via subcutânea, conforme protocolo de imunização descrito a seguir. Como controles, grupos de animais foram imunizados com adjuvante (saponina) ou receberam apenas com PBS.

| GRUPOS | ESQUEMA DE IMUNIZAÇÃO |
|--------------------------|-----------------------------|
| GRUPO 1 (n=17) | Antígeno A2/saponina |
| GRUPO 2 (n=7) | Saponina |
| GRUPO 3 (controle) (n=7) | Tampão salina fosfato (PBS) |

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Após cada dose administrada, foi avaliada a resposta humoral através da determinação pelo método de ELISA dos níveis de anticorpos IgG total, e dos isótipos IgG1 e IgG2, que se constituem em marcadores indiretos da indução de resposta celular do tipo Th2 e Th1, respectivamente. Foram avaliados os níveis de anticorpos, produzidos contra o antígeno vacinal e contra o extrato total de抗ígenos de formas promastigotas totais (método de diagnóstico clássico da LV).

Como pode ser observado nas Figuras 12 e 13, animais vacinados com a formulação vacinal apresentam reação sorológica contra o antígeno vacinal, mas permanecem soronegativos na reação com extrato total de parasitas, sendo possível, portanto, a diferenciação sorológica entre os animais apenas vacinados com A2 daqueles infectados, os quais são soropositivos nos testes de ELISA, em reação com o extrato bruto ou solúvel dos parasitas.

Desta forma, a principal inovação desta formulação vacinal é a sua capacidade de induzir resposta imune humoral em cães caracterizada pela produção de anticorpos específicos contra o antígeno vacinal, porém, que não reagem com o extrato bruto ou solúvel das formas promastigotas de *Leishmania* nos testes de ELISA ou na reação de imunofluorescência. (dados não apresentados).

A figura 12 apresenta um gráfico com os níveis de anticorpos IgG total, IgG1 e IgG2 em amostras de soro de cães antes e após cada dose das imunizações e após a infecção desafio com *L. chagasi*. As placas de ELISA foram sensibilizadas com a proteína recombinante A2 (250 ng) ou com LcPA de *L. chagasi* (1 µg/well). A

produção de IgG total foi determinada por ELISA direta e a de IgG1 e IgG2 por ELISA indireta. O eixo das ordenadas representa a absorbância, em comprimento de onda de 492nm. No eixo das abscissas, cada barra indica a média da produção de IgG total e das subclasses IgG1 e IgG2 acrescido ou subtraído do desvio padrão de cada

5 grupo.

A parte da invenção aqui descrita refere-se à produção de IFN- α em resposta ao antígeno vacinal. A resposta imune celular foi avaliada por meio da dosagem de IFN- γ , por ELISA de captura, após a coleta e o cultivo de células mononucleares do sangue periférico (PBMC) *in vitro* e seu estímulo com a proteína recombinante A2-HIS ou com 10 o extrato solúvel de *L. chagasi* (LcPA). As células também foram estimuladas com concanavalina A (ConA), como controle da viabilidade celular. Adicionalmente, foi feito um controle basal, no qual as células não foram estimuladas.

Conforme observado na figura 13, os animais imunizados com a formulação vacinal antígeno A2/saponina, produziram níveis significativamente maiores de IFN- γ , 15 em cultivos de células estimuladas com a proteína recombinante A2, em relação aos grupos "Controle" ($p = 0,003926799$) e "Adjuvante" ($p=0,018896129$), sob o mesmo estímulo. Comparando a produção de IFN- γ entre os grupos de cães avaliados nos cultivos de células sem estímulo, estimuladas com LcPA e com ConA não foram encontradas diferenças estatísticas significativas. Adicionalmente, a produção de IFN- γ , 20 foi significativamente maior nas células estimuladas com a proteína A2-HIS, quando comparadas àquelas sem estímulo ($p = 0,001374901$), estimuladas com ConA ($p=0,00199055$) ou com LcPa ($p = 0,015277637$), avaliando-se o grupo imunizado com a formulação vacinal antígeno A2/saponina. Diferenças estatísticas significativas não 25 foram encontradas dentro dos grupos "Adjuvante" e "Controle" sob os diferentes estímulos (Figura 13).

Os animais do grupo "Vacinado" apresentaram uma produção significativamente maior de IgG2, específico à rA2, quando comparado à IgG1, antes e 30 após a infecção desafio com *L. chagasi* ($p=0,0000000004$ e $p=0,0000000615$, respectivamente). Isto também foi observado no grupo "Controle", após a infecção desafio ($p=0,0341668528$). Cães do grupo "Vacinado", antes e após a infecção desafio, apresentaram a razão IgG1/IgG2 menor que 1, que é um indicativo de resposta T_H1 (0,12224007 e 0,1085985, respectivamente) (Tabela 1). Já os cães do grupo "Adjuvante" e "Controle", produziram quantidades muito baixas desses anticorpos (Figura 12) e as razões IgG1/IgG2, antes e após a infecção desafio, são

2,1096344 e 0,52443, no grupo "Adjuvante" e são 0,9171905 e 0,4106352, no grupo "Controle" (Tabela 1).

| Grupos avaliados | Antígeno Sensibilizador | |
|------------------|-------------------------|----------------|
| | Antes do desafio | Após o desafio |
| Vacinado | 0,122241 | 0,108599 |
| Adjuvante | 2,109744 | 0,52443 |
| Controle | 0,917787 | 0,410635 |

- 5 Tabela 1: Razão entre as sub-classes IgG1:IgG2 em amostras de soros de cães imunizados com a proteína rA2 (Vacinado) e nos grupos Adjuvante e Controle (PBS), antes e após a infecção desafio (período referente a 1 mês) com *L. chagasi*. As placas foram sensibilizadas com a proteína recombinante A2-HIS (250ng) ou com o LcPA de *L. chagasi* (1µg/well). A produção das subclasses IgG1 e IgG2 foi determinada por ELISA indireta.
- 10

Estes resultados indicam que a formulação vacinal induz o desenvolvimento de uma resposta TH1, que se correlaciona com perfil observado em cães assintomáticos ou resistentes a infecção (Pinelli et al., 1994; Quinnell et al., 2001; Santos-Gomes et al., 2002).

15 A Figura 13 traz um gráfico representando a produção de IFN-γ por células mononucleadas do sangue periférico (PBMC) de cães da raça Beagle imunizados com formulação vacinal antígeno A2/saponina, antes da infecção desafio por *L. chagasi*. As células foram cultivadas (1×10^6 /mL) em 1mL de meio DEMEM completo e estimuladas com a proteína recombinante A2 (10 µg/mL) e com o LcPA de *L. chagasi*, na concentração de 50 µg/mL. Como controles, foram avaliadas as células não estimuladas (Meio). Também foi utilizado como controle a concanavalina A (2,5 µg/mL), um mitógeno, para avaliar a viabilidade celular. Os cultivos foram incubados em estufa, a 37°C com 5% de gás carbônico. Após 48 horas, o sobrenadante foi coletado e a produção de IFN-γ foi determinada por ELISA de captura. No eixo das ordenadas está representado a concentração, em pg/mL, de IFN-γ. No eixo das abscissas, estão os grupos de cães, sendo que "Vacinado" corresponde ao grupo de cães que receberam a formulação vacinal antígeno A2/saponina, "Adjuvante" corresponde ao grupo que recebeu apenas a saponina adjuvante e "Controle" a o grupo que

30

recebeu PBS. Cada barra representa a média da produção de IFN- γ juntamente com o desvio padrão de cada grupo. * indica diferença significativa entre os grupos Vacinado e Adjuvante (A2-HIS) $p= 0,018896129$. ** indica diferença significativa entre os grupos Vacinado e Controle (A2-HIS) $p= 0,003926799$; teste t de Student (Excel).

REIVINDICAÇÕES

- 1. VACINA RECOMBINANTE CO NTRA A LEISHMANIOSE VI SCERAL CANINA CONTENDO O ANTÍGENO A2 E QUE PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE ANIMAIS INFECTADOS,** caracterizada pelo uso em preparações vacinais do antígeno A2 do estágio amastigota de espécies de *Leishmania*, em qualquer forma de administração no hospedeiro, que induz a produção de anticorpos não reativos aos testes de diagnóstico sorológicos convencionais, tendo em vista que estes utilizam抗ígenos de formas promastigotas de *Leishmania*.
- 2. VACINA RECOMBINANTE CO NTRA A LEISHMANIOSE VI SCERAL CANINA CONTENDO O ANTÍGENO A2 E QUE PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE ANIMAIS INFECTADOS,** de acordo com a reivindicação 1, caracterizada por induzir em cães resposta humoral, por meio da produção de anticorpos específicos contra o antígeno vacinal que não reagem com抗ígenos de formas promastigotas de *Leishmania*, formas estas utilizadas nos testes sorológicos convencionais para detecção da infecção.
- 3. VACINA RECOMBINANTE CO NTRA A LEISHMANIOSE VI SCERAL CANINA CONTENDO O ANTÍGENO A2 E QUE PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE ANIMAIS INFECTADOS,** de acordo com a reivindicação 1, caracterizada por induzir, em cães, resposta imune celular associada a resistência à infecção, pela indução de níveis elevados de IFN- γ .
- 4. VACINA RECOMBINANTE CO NTRA A LEISHMANIOSE VI SCERAL CANINA CONTENDO O ANTÍGENO A2 E QUE PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE ANIMAIS INFECTADOS,** de acordo com a reivindicação 1, caracterizada por uma formulação vacinal composta pelo antígeno A2 do estágio amastigota de espécies de *Leishmania*, em todas as suas formas de administração no hospedeiro, combinado com adjuvante

5. VACINA RECOMBINANTE CONTRA A LEISHMANIOSE VISCERAL
CANINA CONTENDO O ANTÍGENO A2 E QUE PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE ANIMAIS INFECTADOS, de acordo com a reivindicação 4,

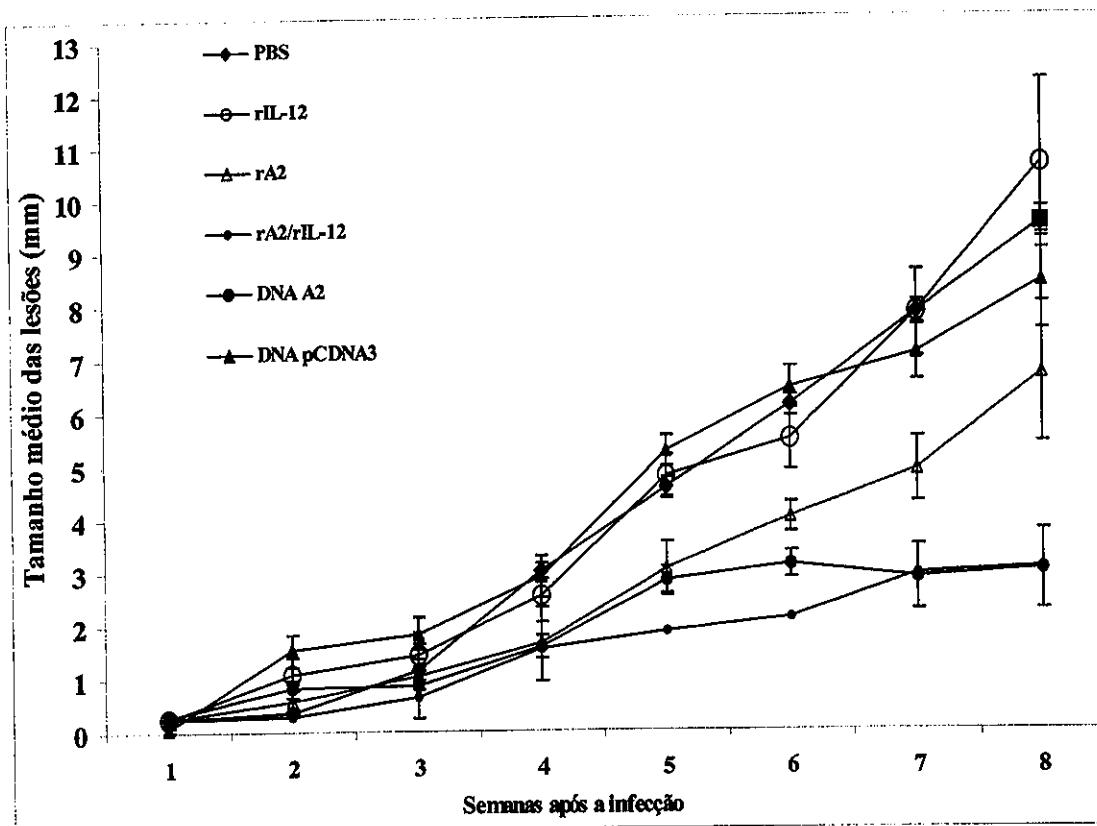
5 caracterizada por uma formulação vacinal contendo proteína recombinante A2-HIS, saponina, solução salina tamponada q.s.p. e Thimerosol, sendo este último utilizado para preservar a proteína.

6. VACINA RECOMBINANTE CONTRA A LEISHMANIOSE VISCERAL
CANINA CONTENDO O ANTÍGENO A2 E QUE PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE

10 ANIMAIS INFECTADOS, caracterizada pela manutenção da soronegatividade dos cães aos testes sorológicos convencionais após a administração de cada uma das doses vacinais, tornando possível a diferenciação sorológica entre os animais vacinados com A2 da forma amastigota de *Leishmania* daqueles infectados.

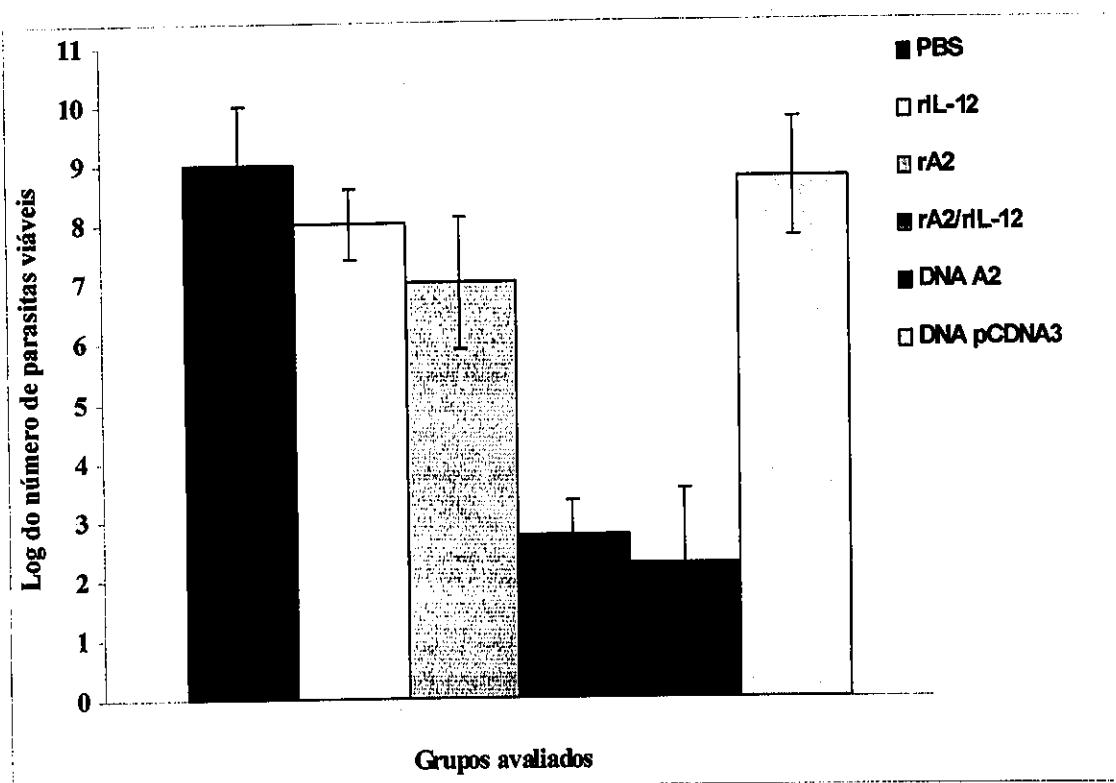
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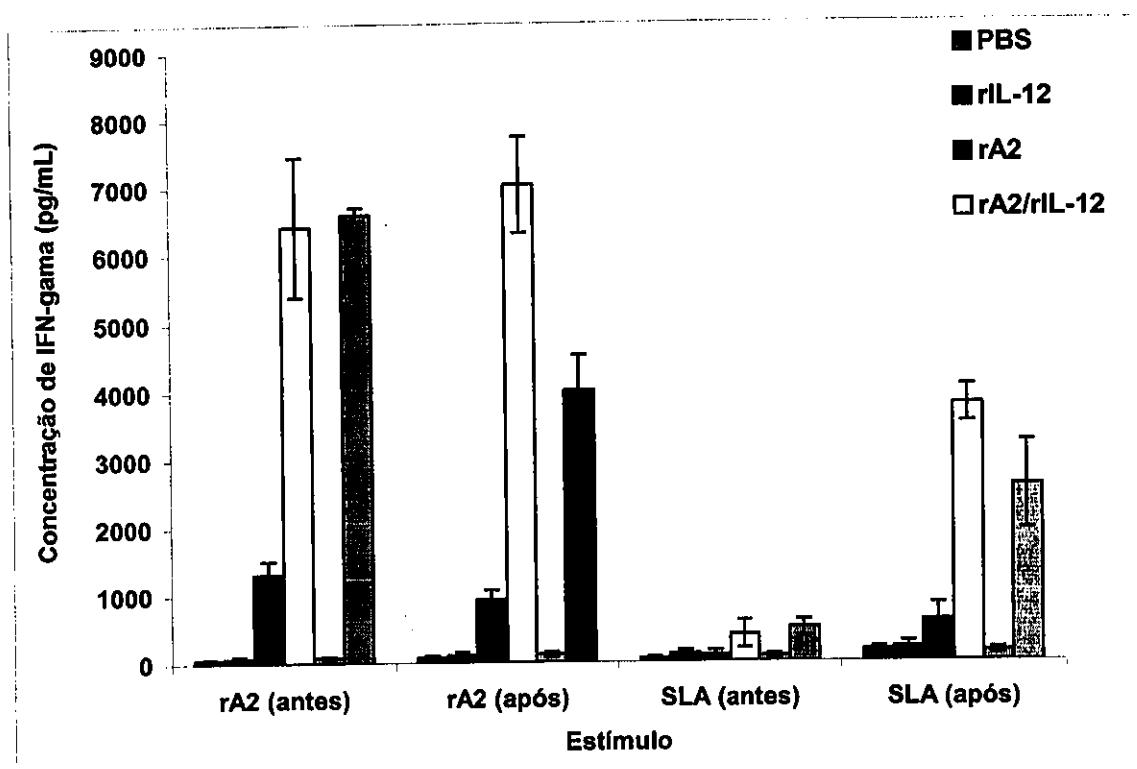
Figura 1

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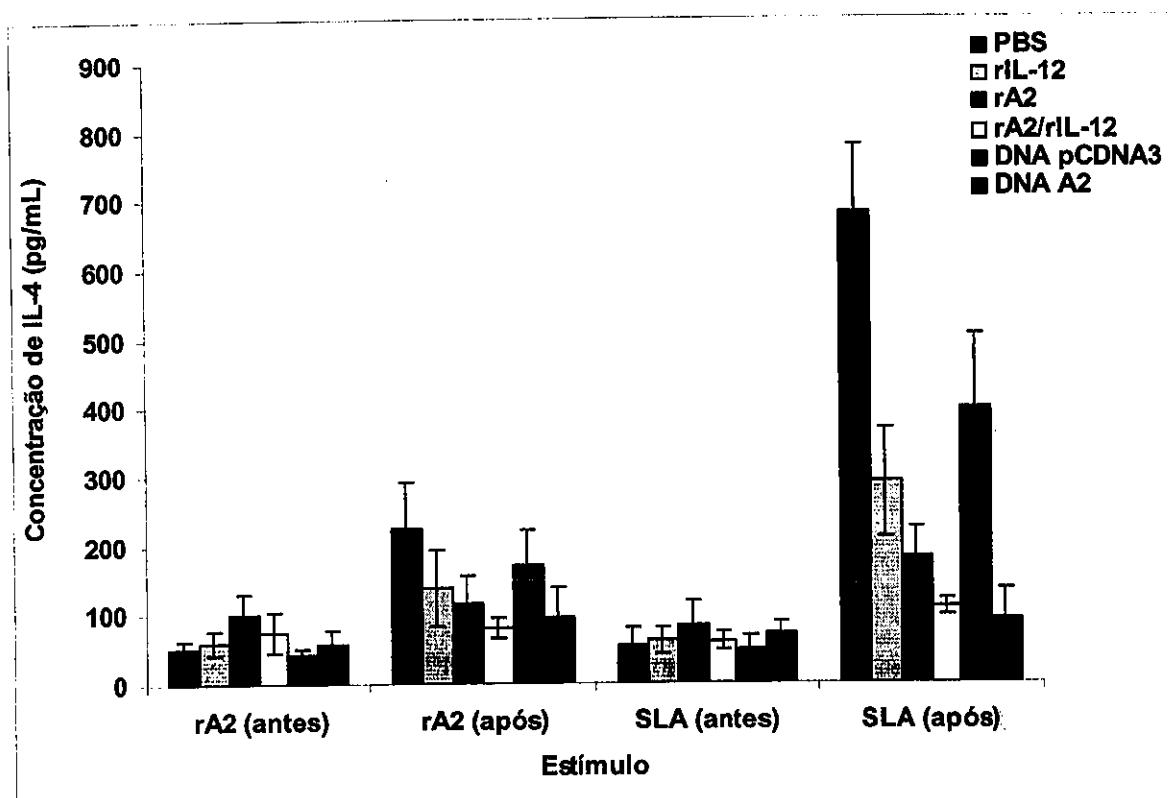
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Figura 2

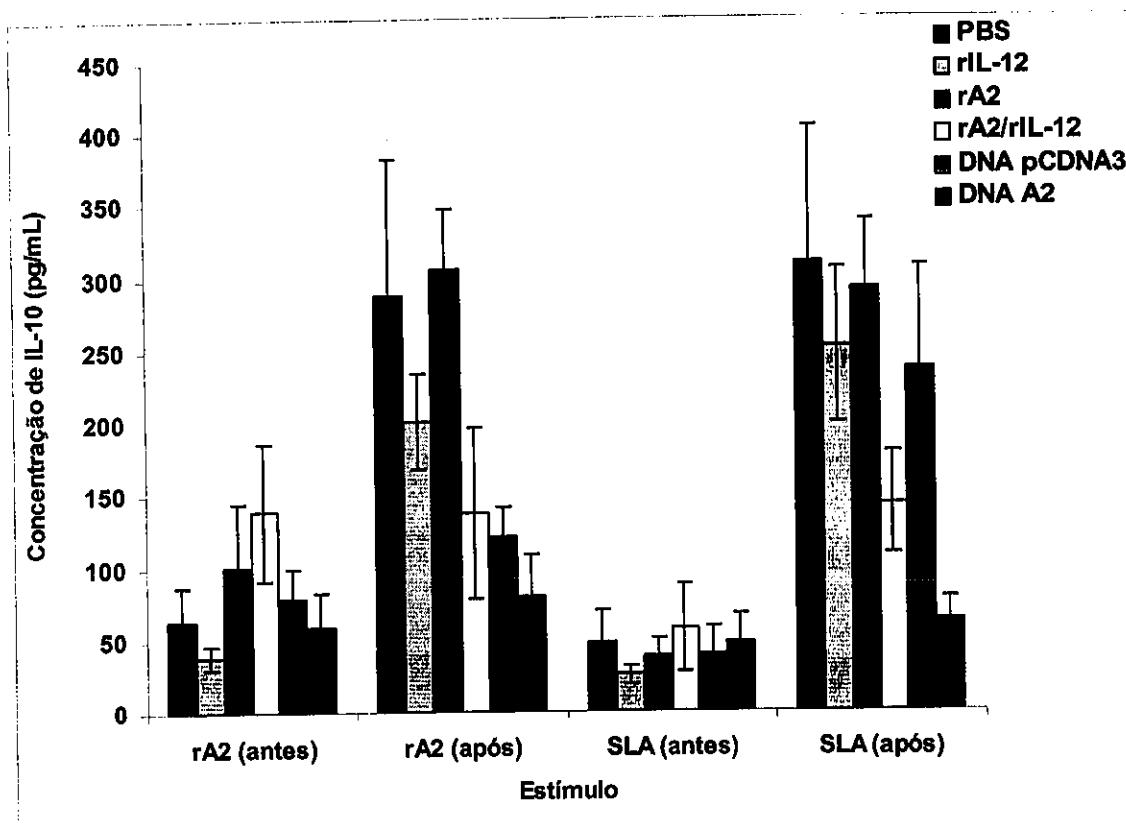
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Figura 3

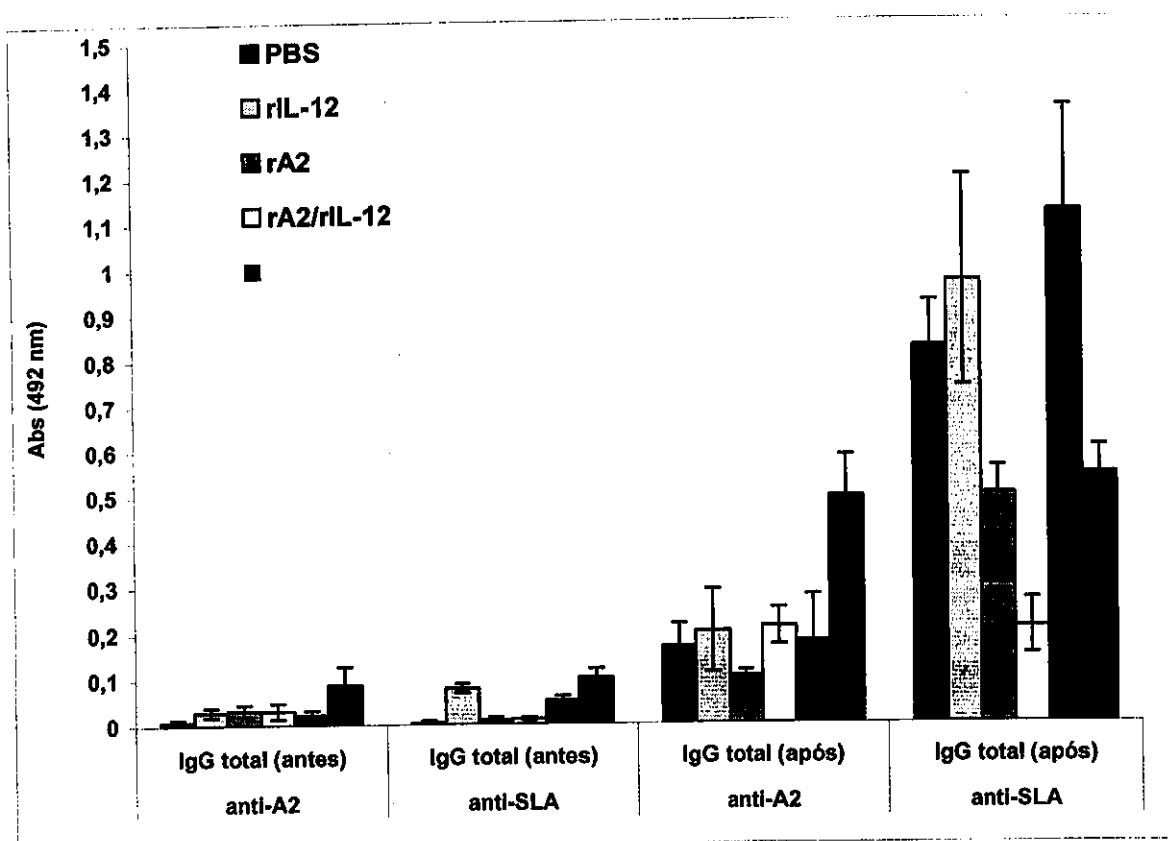
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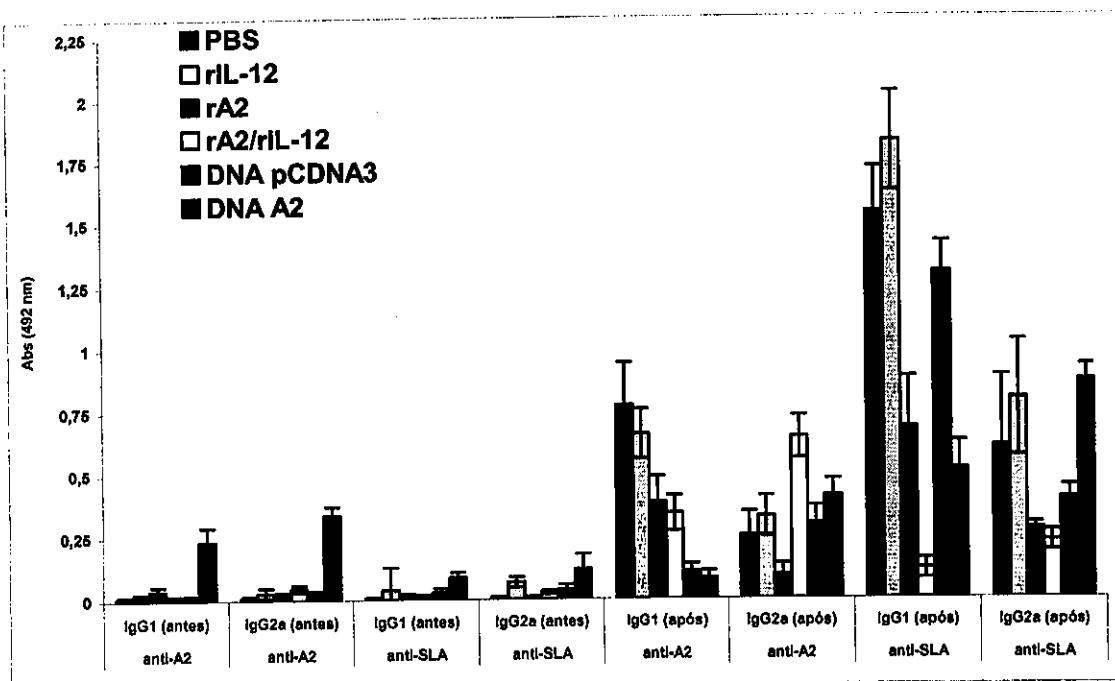
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Figura 5

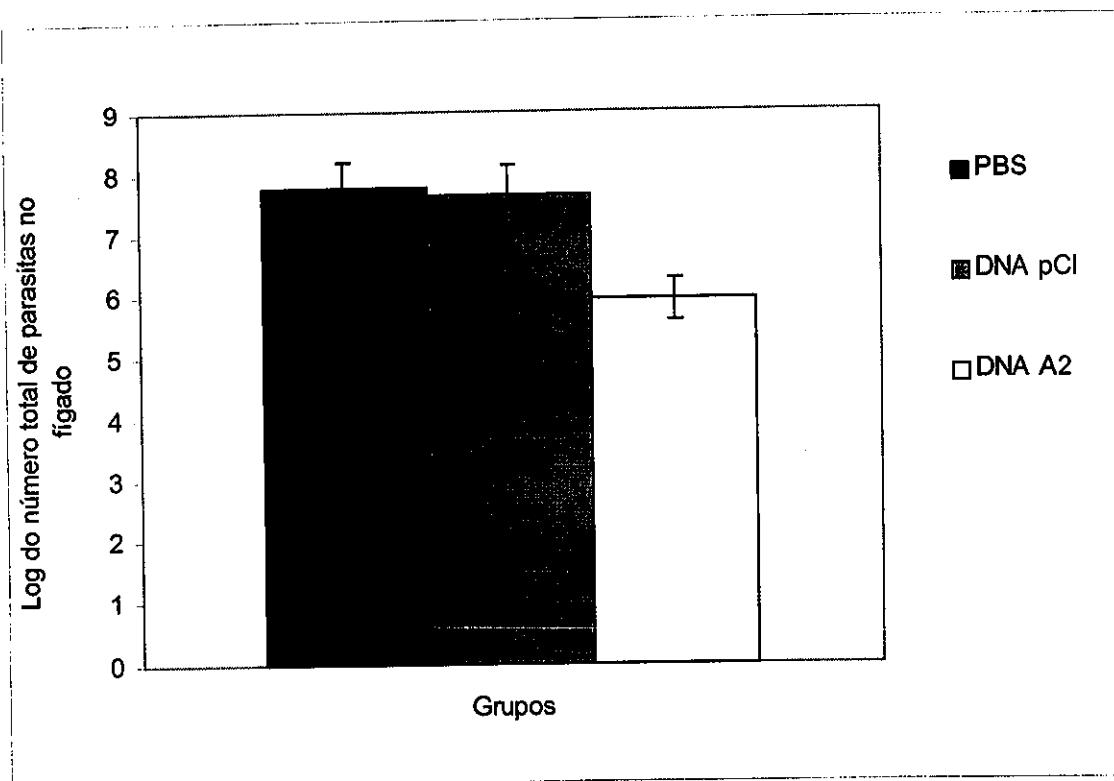
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Figura 6

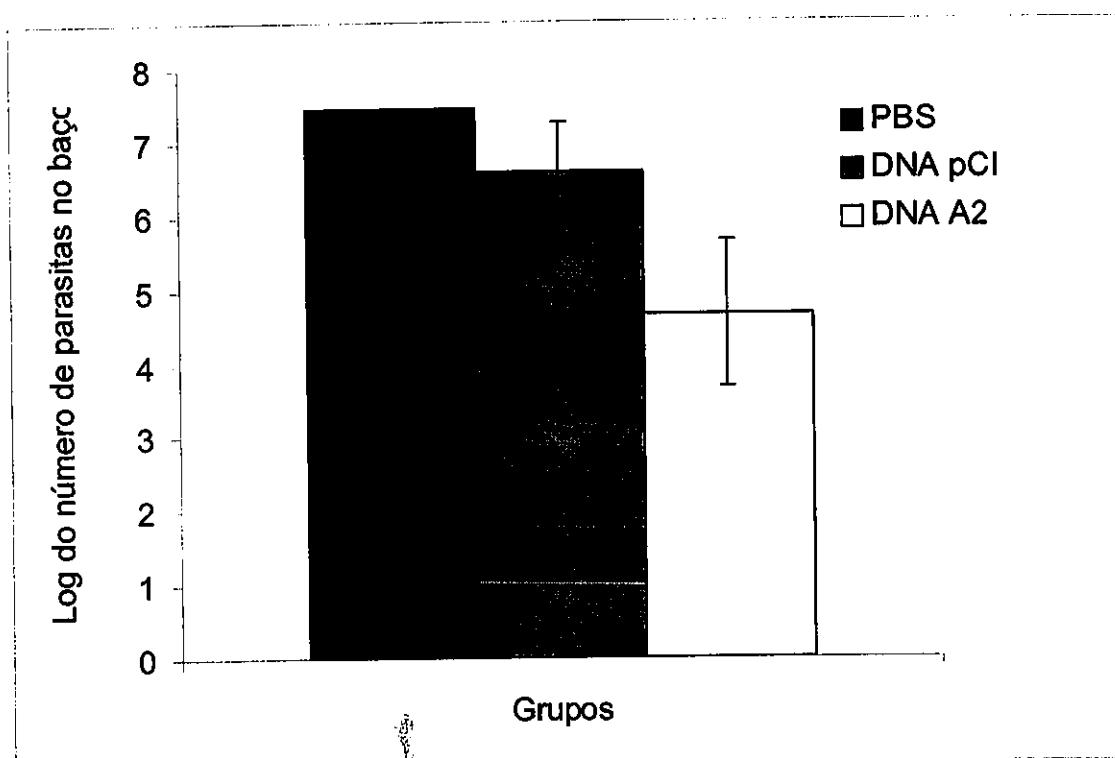
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Figura 7

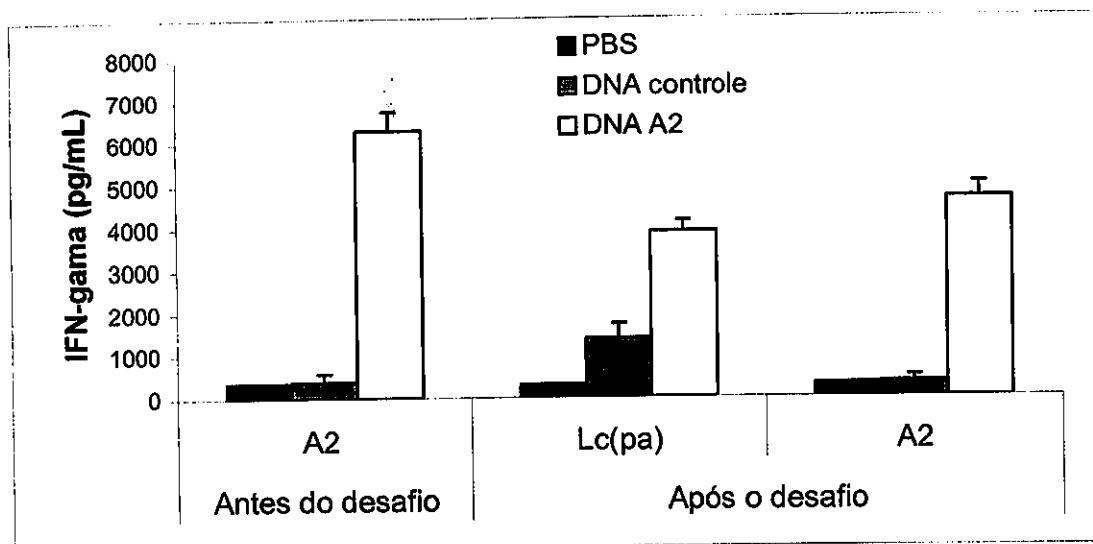
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Figura 8

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Figura 9

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Figura 10

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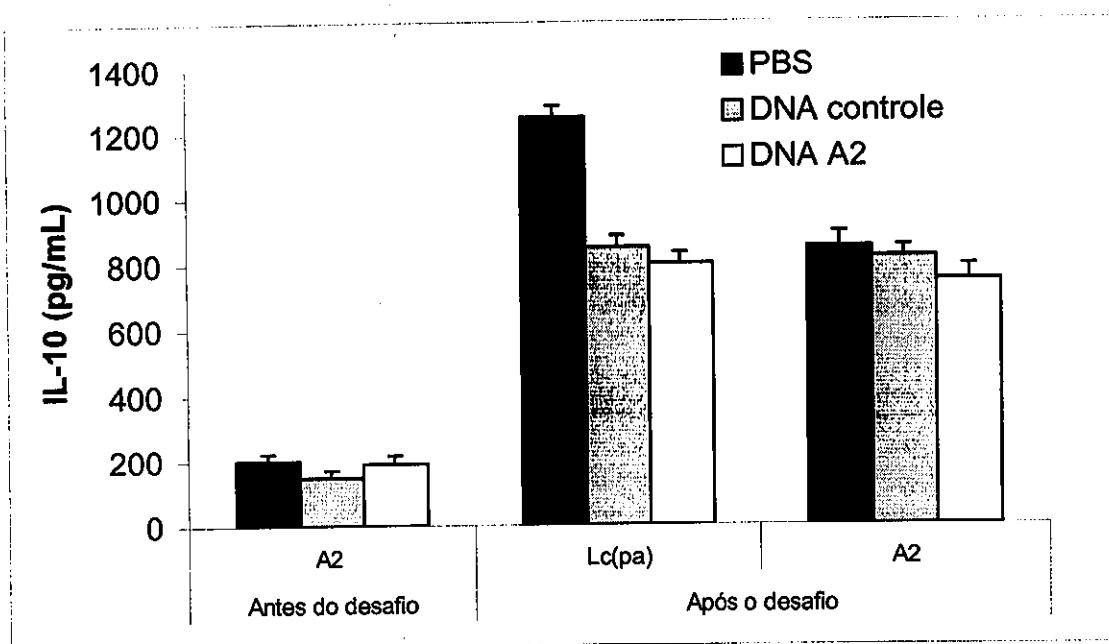
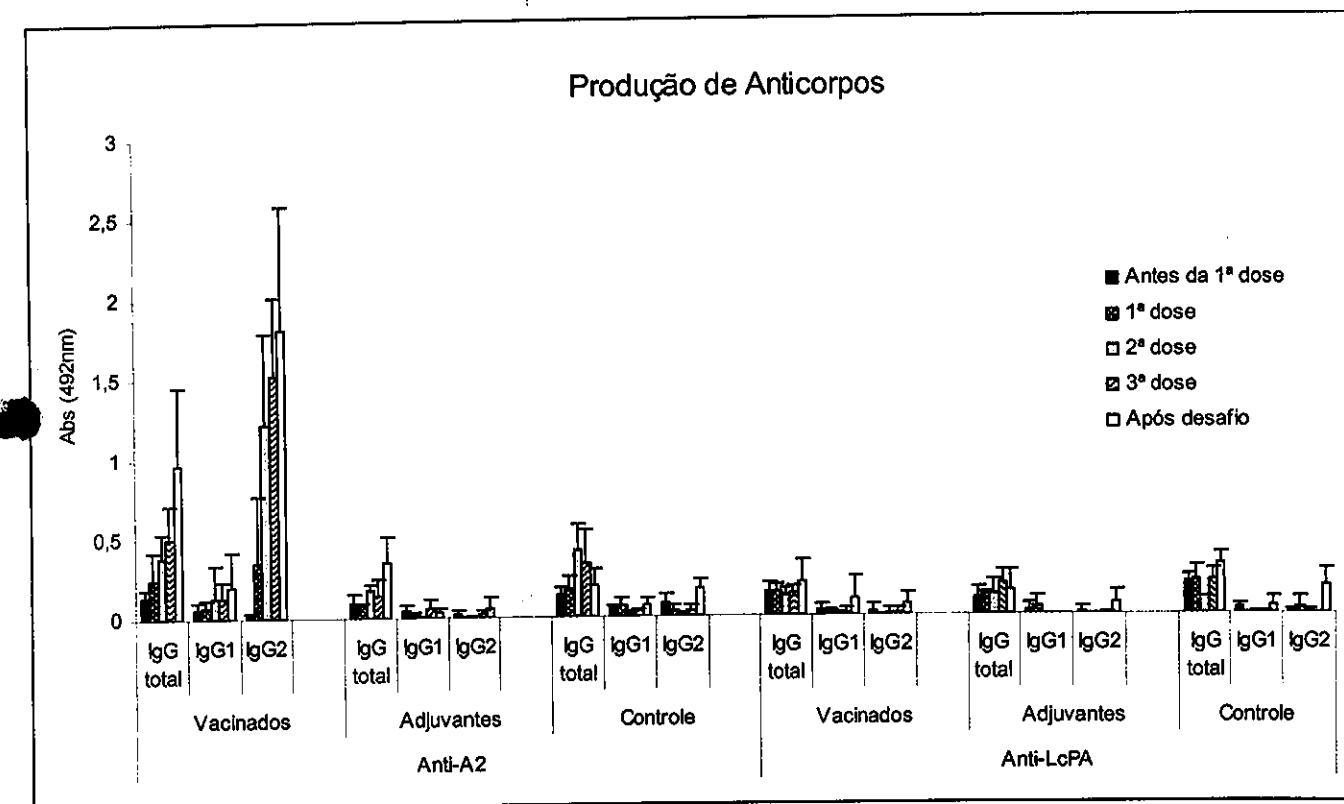
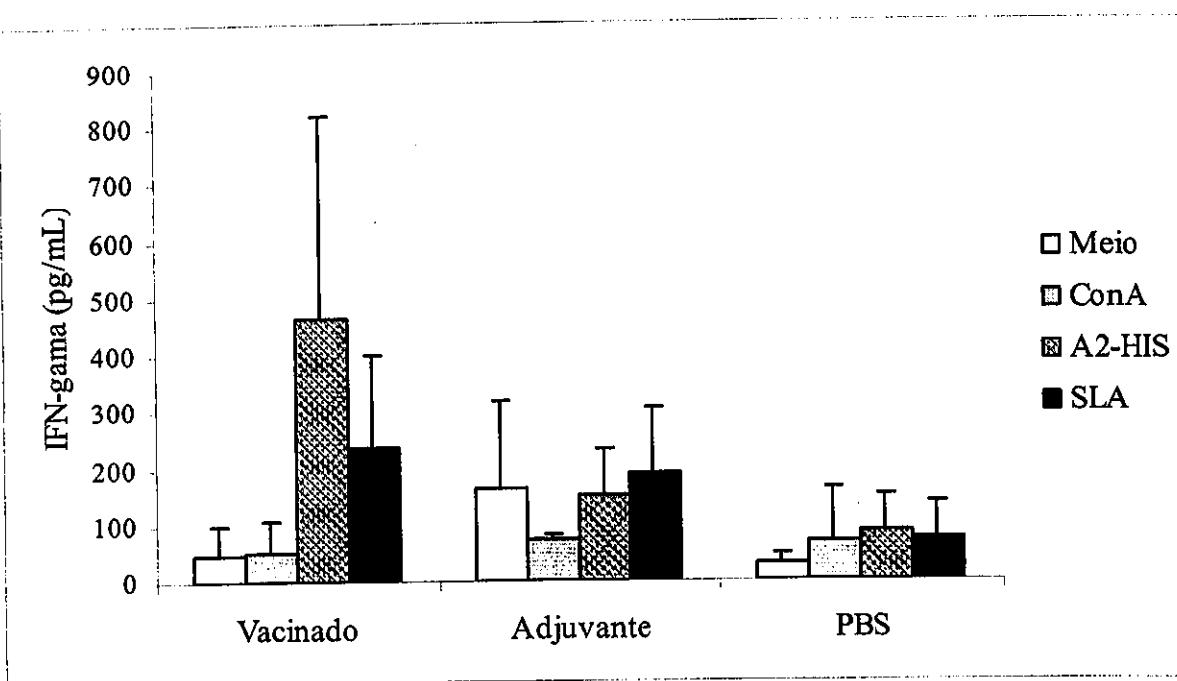
Figura 11

Figura 12

vs

Figura 13

*lhe***RESUMO**

**PROCESSO PARA VACINA RECOMBINANTE CONTRA A LEISHMANIOSE
VISCERAL CANINA CONTENDO O ANTÍGENO RECOMBINANTE A2 E QUE
PERMITE A DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS VACINADOS DE
ANIMAIS INFECTADOS.**

A presente invenção refere-se à vacina recombinante contra a leishmaniose visceral canina contendo a proteína recombinante A2 e saponina, como adjuvante, e que permite a distinção entre animais vacinados e infectados por meio de testes de ELISA ou imunofluorescência convencionais que empregam antígenos de formas promastigotas de *Leishmania*. A vacina objeto do presente pedido caracteriza-se pela manutenção da soronegatividade dos cães aos testes sorológicos convencionais após a administração de cada uma das doses vacinais, tornando possível a diferenciação sorológica entre os animais vacinados com A2 da forma amastigota de *Leishmania* daqueles infectados.

PI0603490

EXAME PRELIMINAR (PI - C - MU)

1- Petição de Depositante: (MG) , de 21/07/06.

014060008038

2- Depositante: Universidade Federal de Minas Gerais

2.1- Procurador: —

3- Natureza: (X) PI () C () MU

4- Título Resumido: Processo para vacina (...)

5- Condições do Pedido:

| | S | N |
|---|---|---|
| Requerimento de depósito com os campos obrigatórios preenchidos | / | |
| Idioma Português | / | |
| Número suficiente de cópias (3) | / | |
| Relatório Descritivo | / | |
| Reivindicações | | / |

| | S | N |
|---|---|---|
| PI e C – Apresenta desenhos citados ou não cita nem apresenta desenhos. | / | |
| MU Desenhos | | / |
| Resumo | | / |
| Datilografado no padrão exigido | / | |
| Valor Correto de Recolhimento | | / |

6- Resultado do Exame: O pedido atende formalmente as disposições legais especialmente quando ao Art. 19 da LPI e Ato Normativo 127/97 estando apto a ser protocolizado.

Em, 18/08/06.

Assinatura e Carimbo
Anna Carla de Mello Rocha
Técnico I - Mat. 1467187
DIRPA/SEXAME

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JUNTADA A PETIÇÃO N.º 01/002248

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De: SEPPCT
A: SEPDOC

Solicito a confecção e remessa a este setor da cópia oficial do pedido PE-0603430 - 0
a fim de instruir o pedido internacional PCT/BR 2007 000248 requerido pelo
depositante no formulário PCT/RO/101.

Rio, 03 OUT 2007

SEPPCT

Marcia Timóteo da Costa
Chefe da DIRPA SEPPCT
Mat. 448392

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Título (54) : PROCESSO PARA VACINA RECOMBINANTE
CONTRA A LEISHMANIOSE VISCERAL
CANINA CONTENDO O ANTÍGENO
RECOMBINANTE A2 E QUE PERMITE A
DISTINÇÃO SOROLÓGICA ENTRE ANIMAIS
VACINADOS DE ANIMAIS INFECTADOS

Nome do Depositante (71) : Universidade Federal de Minas Gerais (BR/MG)

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de Freitas Abrantes, Eduardo Antonio Ferraz
Coelho, Ricardo Tostes Gazzielli

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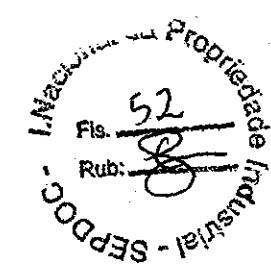
JUNTADA DE PETIÇÃO

Foram juntadas, nesta data, as seguintes petições:

| Petição | Data de protocolização |
|--------------------|------------------------|
| mº 014090003560/MG | 17/07/2009 |
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Publicação, em 28/10/2010

CARLA FERNANDES DE FREITAS
TÉCNICO EM PI - MAT. 1489558
SEPDOC/PUB



Developmental Gene Expression in *Leishmania donovani*: Differential Cloning and Analysis of an Amastigote-Stage-Specific Gene

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Leishmania protozoans are the causative agents of leishmaniasis, a major parasitic disease in humans. During their life cycle, *Leishmania* protozoans exist as flagellated promastigotes in the sand fly vector and as nonmotile amastigotes in the mammalian hosts. The promastigote-to-amastigote transformation occurs in the phagolysosomal compartment of the macrophage cell and is a critical step for the establishment of the infection. To study this cytodifferentiation process, we differentially screened an amastigote cDNA library with life cycle stage-specific cDNA probes and isolated seven cDNAs representing amastigote-specific transcripts. Five of these were closely related (A2 series) and recognized, by Northern (RNA) blot analyses, a 3.5-kb transcript in amastigotes and in amastigote-infected macrophages. Expression of the amastigote-specific A2 gene was induced in promastigotes when they were transferred from culture medium at 26°C and pH 7.4 to medium at 37°C and pH 4.5, conditions which mimic the macrophage phagolysosomal environment. A2 genes are clustered in tandem arrays, and a 6-kb fragment corresponding to a unit of the cluster was cloned and partially sequenced. An open reading frame found within the A2-transcribed region potentially encoded a 22-kDa protein containing repetitive sequences. The recombinant A2 protein produced in *Escherichia coli* cells was specifically recognized by immune serum from a patient with visceral leishmaniasis. The A2 protein repetitive element has strong homology with an S antigen of *Plasmodium falciparum*, the protozoan parasite responsible for malaria. Both the A2 protein of *Leishmania donovani* and the S antigen of *P. falciparum* are stage specific and developmentally expressed in mammalian hosts.

Leishmania protozoans are the causative agents of human leishmaniasis, which includes a spectrum of diseases ranging from self-healing skin ulcers to fatal visceral infections. *Leishmania donovani* causes visceral leishmaniasis, also known as kala-azar, which has a high mortality rate if not treated. The *Leishmania* protozoans exist as extracellular flagellated promastigotes in the alimentary tract of the sand fly and are transmitted to the mammalian hosts through the bite of the insect. Once injected through the skin, the promastigotes are taken up by macrophages, rapidly differentiate into amastigotes, and start to multiply within the phagolysosomal compartment. As the infected cells rupture, amastigotes subsequently infect other macrophages, giving rise to the various symptoms associated with leishmaniasis (23).

While in the midgut of the insect, newly transformed promastigotes, functionally avirulent, progressively acquire capacity for infection and migrate to the mouthparts (30, 32). This process, termed metacyclogenesis, is concurrent with the differential expression of major surface glycoconjugates which mediate the migration of promastigotes in the alimentary tract and prepare the organism for the serum environment (27, 28, 31, 33). In comparison, the promastigote-to-amastigote cytodifferentiation is a profound morphological and physiological transformation. During differentiation, the parasite loses its flagellum, rounds up, changes its glycoconjugate coat (13, 21, 22, 39), and starts to express a set of metabolic enzymes optimally active at low pHs (14, 25). The survival of the parasite inside the macrophage phagolysosome is associated with its ability to down-regulate many effector and accessory

functions of its host cell, including oxygen metabolite-mediated killing and the capacity of the macrophage to act as an efficient antigen-presenting cell (5, 20). Glycoconjugates that coat the promastigote have been shown to play a critical role in the mechanism of entry of the parasite into its host cell and in the modulation of the host immune response (reviewed in references 24 and 38).

Because of difficulties in obtaining a sufficient number of pure and viable amastigotes, the biology of this form of the parasite has not been well characterized. The importance of the promastigote-amastigote cytodifferentiation for the establishment of infection in the mammalian host has prompted us to identify molecular events involved in this process. Our approach was to identify *Leishmania* genes developmentally expressed during the amastigote stage. Our assumption was that genes expressed only by amastigotes were likely to be essential for the survival of the parasite in its human host.

In this study, we have characterized an amastigote-stage-specific gene (A2 gene) and determined the physiological conditions required to induce its expression. We also demonstrate that the A2 gene product is recognized by serum from a kala-azar patient and has identity with a major antigen developmentally expressed by the pathologic forms of the malaria-associated protozoan *Plasmodium falciparum*. These results suggest that the *Leishmania* and *Plasmodium* parasites, phylogenetically distant parasitic protozoans, have evolved the A2-like genes to carry out similar functions associated with infecting their human hosts.

MATERIALS AND METHODS

* Corresponding author. Mailing address: Institute of Parasitology of McGill University, Macdonald Campus, 21,111 Lakeshore Rd., Ste-Anne de Bellevue, Québec, Canada H9X 3V9.

Leishmania strains and media. Amastigotes of the *L. donovani* infantum Ethiopian LV9 strain were harvested from

spleens of infected female gold Syrian hamsters and purified as described by Reiner (29). Briefly, parasites were released from tissue with a homogenizer, the mixture was centrifuged three times at $100 \times g$ to remove cellular debris, and amastigotes were pelleted at $1,500 \times g$. The pellet was resuspended in 0.17 M sodium acetate to lyse contaminating erythrocytes. The amastigotes were recovered by centrifugation and were incubated at 37°C in complete RPMI medium (RPMI 1640 supplemented with 10% endotoxin-free heat-inactivated fetal bovine serum, 10 ml of 1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.3], 100 U of penicillin per ml, 100 U of streptomycin per ml) for 18 h prior to RNA extractions. In preliminary analyses, we observed that a period of incubation of 18 h and multiple washes were necessary to allow the amastigote to dislodge completely from host cell debris and then to obtain amastigote RNA samples relatively free of mammalian RNA contamination. To obtain promastigotes, LV9 strain amastigotes were allowed to differentiate in complete RPMI medium at 26°C and cultured for at least 7 days in the same medium before use. Promastigotes of the *L. donovani* Sudanese strain 1S2D (wt) were cultivated and passaged in complete RPMI medium at 26°C . Amastigote-like organisms of the 1S2D strain were cultivated by procedures described by Doyle et al. (12).

The Sudanese strains 1S2D and 1S2D (wt) were a gift from S. Turco (University of Kentucky, Lexington). The 1S2D (wt) promastigotes were adapted to grow in axenic conditions and lost the ability to transform into infective promastigotes (37b).

Nucleic acid preparations. Total RNA of amastigotes and promastigotes was prepared by the guanidinium isothiocyanate method with RNAzol (Cinna/Biotex Laboratories International, Inc., Friendswood, Tex.); poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography (grade 7; Pharmacia) as described by Sambrook et al. (34). *Leishmania* total DNA was extracted from promastigotes by the methods described for mammalian cells by Sambrook et al. (34).

Construction and screening of an amastigote cDNA library. A total of 10 μg of amastigote mRNA was used to construct an EcoRI-XhoI unidirectional cDNA library of 10^6 clones in the λ ZAP II vector (Stratagene); hemimethylated cDNA was produced with manufacturer-supplied reagents and protocols. About 40,000 clones of the primary library were screened differentially with stage-specific probes; the cDNA probes were prepared with oligo(dT)12–18 primer (Pharmacia) and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories [BRL]) by protocols described by Sambrook et al. (34). Duplicate filters were hybridized with each probe for 18 h at 42°C in 50% formamide– $6 \times$ SSC–5% Denhardt's solution ($1 \times$ SSC consists of 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0; $1 \times$ Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin)–5% dextran sulfate. Membranes were then washed twice at room temperature in $1 \times$ SSC for 20 min, washed twice at 55°C in $1 \times$ SSC–0.1% sodium dodecyl sulfate (SDS), and then autoradiographed on Kodak X-Omat films with an intensifying screen for 18 to 72 h. Areas on the plates containing putative clones of interest were picked, and the phage pools were submitted to a second round of screening. Only clones which were hybridizing with the amastigote cDNA probe, but not with the promastigote probe, were considered. For each cDNA clone isolated, a Bluescript plasmid derivative was excised from the λ ZAP II recombinant phages *in vivo* with the helper phage R-408 (Stratagene protocol).

Northern and Southern blot analysis. Northern (RNA) blot analyses were carried out as previously described (11); unless specified otherwise, 10 μg of total RNA was loaded in each

lane. For Southern blot analysis, 10 μg of total DNA was digested to completion with restriction enzymes (BRL), and fragments were separated on 0.7% agarose gels and transferred to nylon membranes (Hybond-N; Amersham) as described by Sambrook et al. (34). Membranes were prehybridized for a few hours in the hybridization buffer (50% formamide, $6 \times$ SSC, 5% Denhardt's solution, 0.1% SDS, 200 μg of denatured salmon sperm DNA per ml) before the addition of the probes. Hybridizations were carried out for 18 h at 42°C . The membranes were washed twice in $1 \times$ SSC for 15 min at room temperature, washed twice in 2 to 0.1 \times SSC–0.1% SDS at 55 to 60°C for 15 min, and autoradiographed on Kodak X-Omat films with intensifying screens. DNA probes were prepared with agarose gel-purified restriction fragments labelled to high specificity with [α -³²P]dCTP (ICN; 3,000 Ci/mmol) by nick translation (Amersham kit and protocol).

Isolation of genomic clones. A 6- to 10-kb EcoRI fragment partial genomic library was constructed in the λ ZAP II vector (Stratagene). More than 2,000 clones were screened on duplicate filters with nick-translated probes prepared with the A2 cDNA by techniques and hybridization conditions described for the differential screening. Eight clones were isolated and purified. Bluescript plasmid derivatives were excised from recombinant λ phages as for cDNA clones.

DNA sequencing. The A2 cDNA sequence was determined with oligonucleotide DNA primers. The 5' and 3' ends of A3 to A11 were obtained with common or A2 cDNA-specific primers. For the 1.9-kb *Xho*I-EcoRI portion of the genomic clone GECO 90, the fragment was subcloned in the Bluescript phagemids KS⁺ and KS⁻. Nested deletions were carried out on plasmids with exonuclease III and S1 nuclease (*double-stranded* Nested Deletion Kit; Pharmacia). Sequencing reactions were performed on single-stranded DNA templates (prepared with the M13K07 helper phage and by the method of Sambrook et al. [34]) or double-stranded DNA plasmids with the Deaza T7 sequencing mixes (Pharmacia) and labelled nucleotides (³⁵S-ATP or ³⁵S-CTP; 600 Ci/mmol; Amersham). Reactions were analyzed on 6% denaturing polyacrylamide gels.

Subcloning of the ORFII fragment. Synthetic oligonucleotides containing tails with *Bam*HI restriction sites were used to amplify the ORFII coding region from the 1.9-kb *Xho*I-EcoRI fragment subcloned in a Bluescript KS⁺ subclone (5' primer, ctcgaggatccgatcgaagtccgcacgtgc; 3' primer, tagagaggatccatctatggatcgcttg [underlined sequences correspond to the cloning tails]). Reactions were performed with the *Tth* DNA polymerase (Pharmacia) and the GeneAmp PCR system 9600 (Perkin-Elmer Cetus). After 3 min at 95°C to denature the linearized template, the reaction mixture was first incubated for 15 cycles at 55, 75, and 95°C for 2 min, 3 min, and 30 s, respectively, and the annealing temperature was raised from 55 to 65°C for 20 subsequent cycles. The PCR product (1.1 kb) was first inserted in the PCR II cloning vector (T/A cloning system; Invitrogen) and then subcloned with the *Bam*HI restriction sites in the pET16b expression vector (Novagen), in frame with the histidine tag (HT).

Immunoprecipitations and western blot analysis. The HT-A2 fusion protein was synthesized *in vitro* with T7 RNA polymerase and rabbit reticulocyte lysate (TNT assay; Promega) and labelled with L-[³⁵S]methionine (ICN; >1,000 Ci/mmol). A human p53 cDNA construct was used as a positive control for the *in vitro* transcription-translation assay, and then the labelled p53 protein was used as a negative control for the immunoprecipitations. Precleared protein samples were diluted with 1 volume of NET/Gel (50 mM Tris [pH 7.5], 150

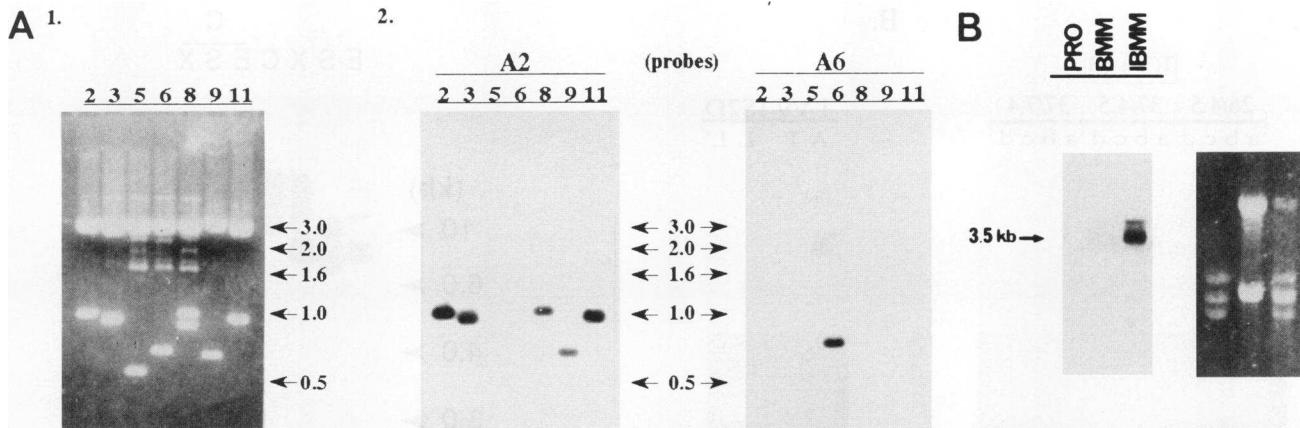


FIG. 1. (A) Southern blot analysis of amastigote-specific cDNA clones. Panel 1, Bluescript SK⁻ recombinant plasmids (A2-A11) were digested with *Eco*RI and *Xba*I to excise the cDNA inserts. Fragments were separated on an agarose gel (1%) and revealed by ethidium bromide staining; cDNA inserts varied from 0.5 kb (A5) to 1.8 kb (A8; contained an internal *Eco*RI site). Panel 2, Restriction fragments were transferred to nylon membranes, and duplicates were hybridized with a cDNA clone A2 (0.9 kb)- or A6 (0.6 kb)-specific probe. The A2 probe recognized five cDNAs (A2, A3, A8, A9, and A11); the A6 cDNA hybridized only to itself. (B) Differential expression of A2-related genes in promastigotes and amastigote-infected macrophages. Total RNA (15 µg) extracted from bone marrow-derived macrophages (BMM), *L. donovani* LV9-infected BMM (IBMM), and *L. donovani* LV9 promastigotes (PRO) was analyzed by Northern blot analysis with an A2 cDNA-specific probe (0.9 kb). Murine bone marrow-derived macrophage cultures and *L. donovani* amastigote in vitro infections were carried out as previously described (11). Total RNA samples were stained with ethidium bromide on the gel prior to the transfer (right panel). Note that *Leishmania* rRNA runs as three bands which are present in the promastigote lane and the amastigote-infected macrophage lane (41).

mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40, 0.25% gelatin) and allowed to react with 3 µl of each serum sample for 2 h at room temperature; immune complexes were precipitated with 1 volume of prepared *Staphylococcus aureus* Cowan 1-fixed cells (Immuno-precipitin; BRL). Pellets were washed three times with 1 ml of NET/Gel, and samples were analyzed on SDS-polyacrylamide gels (19). Gels were treated with Amplify reagent (Amersham), dried, and autoradiographed for 18 h on Kodak X-Omat film. The kala-azar immune serum and the negative control serum were provided by the National Center for Parasitology (Serology). Preparation of *E. coli* total proteins and Western blot (immunoblot) analysis were carried out as described previously (19); a 1/100 dilution of the kala-azar L1 serum was used for first antibodies, a biotinylated goat anti-human immunoglobulin G Fc (BRL) was used as the second. Bound antibodies were detected with streptavidin-conjugated horseradish peroxidase (Amersham) and 3,3'-diaminobenzidine (Sigma) in a 0.03% H₂O₂ solution.

Processing of the in vitro-synthesized A2 protein. For cleavage of the HT sequence from the HT-A2 fusion protein, aliquots of the TNT reaction samples (2.5 µl) were added to 17.5 µl of factor Xa reaction buffer (20 mM Tris-Cl [pH 8.0], 100 mM NaCl, 2 mM CaCl₂) containing 1 µg of factor Xa protease (NEB). Samples were incubated at 37°C for 90 min; the reaction was stopped by the addition of 10 µl of 3× SDS loading buffer, and samples were boiled for 10 min. The in vitro processing of the A2 protein was analyzed with canine pancreatic microsomal membranes (CPMM; Promega) and in vitro transcription-translation assays; 2.5 µl of CPMM was added to the TNT reactions prior to the addition of T7 RNA polymerase (Promega protocol); proteins were labelled with [³⁵S]cysteine (ICN; >1,000 Ci/mmol) or L-[¹⁴C]leucine (Amersham; 317 mCi/mmol). The plasmid template used in these assays (pET16b/ORFIINX) was constructed by deleting the *Nco*I-*Xba*I fragment (70 bp) containing the HT coding sequence from the pET16b/ORFI plasmid; the original ATG of the A2 protein replaced the ATG of the HT-A2 fusion protein near the Shine-Dalgarno sequence (see Fig. 4, lower panel).

After 90 min of incubation at 30°C, the microsomes were pelleted (10 min at 16,000 × g and 4°C) and washed three times with 100 µl of phosphate-buffered saline (PBS) (pH 7.3). The microsomal fraction was resuspended in 20 µl of PBS; a sample consisted of 12 µl of microsomes added to 6 µl of 3× SDS loading buffer and denatured by boiling.

RESULTS

Isolation of cDNA clones representing amastigote-specific transcripts. The morphological and physiological changes of the *Leishmania* parasite which occur following its transfer from the sand fly to a mammalian host suggest a rapid modulation of the expression of numerous genes. To identify molecular events associated only with the amastigote stage, our approach was to compare the gene expression of both forms of the parasite and characterize transcripts developmentally expressed in amastigotes. We constructed an amastigote cDNA library from amastigotes purified from infected animals and differentially screened about 40,000 clones with stage-specific cDNA probes. This strategy yielded seven independent clones which hybridized only with the amastigote-specific probes. For each of these, a pBluescript plasmid derivative was excised from the λ ZAP II cloning vector. The cDNAs were sized on agarose gels and compared with each other by Southern blot analysis. As shown in Fig. 1A, five of these cDNA clones (A2, A3, A8, A9, and A11) were recognized by the A2 cDNA probe and, therefore, represented the same or closely related transcripts. The A6 and A5 cDNAs were the only representatives of their respective genes.

We verified the developmental expression of the transcripts in the amastigote stage by Northern blot analysis. A probe prepared with the cDNA insert of the A2 plasmid (0.9 kb) recognized predominantly a 3.5-kb transcript which was present in amastigote-infected macrophages but not in promastigotes or in noninfected macrophages (Fig. 1B). This confirmed the developmental expression of the A2-related genes in amastigote-infected macrophages. On longer expo-

A.



B.



FIG. 2. (A) Induction of A2 gene expression in promastigotes. Promastigotes of the *L. donovani* Sudanese 1S2D (wt) strain cultivated in complete RPMI medium at 26°C and pH 7.4 were transferred into media at pH 4.5 (HEPES in complete RPMI was replaced by 20 mM succinic acid) and incubated at 26 or 37°C or were maintained in medium at pH 7.4 but incubated at 37°C, as indicated above the lanes. Total RNA was extracted after 3, 6, 10, and 30 h following the transfer (lanes a, b, c, and d, respectively). (B) Induction of A2 gene expression in in vitro-cultured amastigote-like organisms (*L. donovani* Sudan strain 1S2D). Amastigotes were transferred in fetal bovine serum supplemented with 20 mM succinate for 18 h to reduce the pH (L') or were maintained under neutral pH conditions (L). Both L and L' cells were incubated at 37°C. Lanes P and A, RNA samples from *L. donovani* Ethopian LV9 promastigotes and amastigotes isolated from infected hamsters, respectively. For both panels, RNA samples were analyzed by Northern blot with A2 cDNA-specific probes (0.9 kb). Equal loading of RNA in each lane was verified by ethidium staining of the agarose gel prior to the Northern blot.

sure, however, weak hybridization bands were observed for the promastigote RNA, indicating that expression was not totally shut off in promastigotes (data not shown). The A5 and A6 cDNAs also recognized RNAs present only in amastigotes; A5 hybridized with a high-molecular-weight transcript (8.0 kb), and A6 hybridized with a 1.0-kb RNA (not shown). These cDNAs have not as yet been characterized.

Physicochemical factors triggering the expression of the amastigote-specific genes. The promastigote-to-amastigote cytodifferentiation process is thought to be triggered by temperature and pH shifts occurring during the passage from insect vector to mammalian host. The insect alimentary tract is at 27°C and neutral pH, whereas the macrophage phagolysosome compartment is at 37°C and pH 4.5 (2). We investigated the importance of both of these physicochemical parameters on the induction of expression of the A2 transcripts. We attempted to induce the expression of the amastigote-specific A2 gene in cultured promastigotes by shifting the temperature and pH of the culture media. Promastigotes of *L. donovani* Sudanese strain 1S2D (wt) cultured at 26°C and at pH 7.4 were transferred into media at pH 4.5 and/or 37°C, and total RNA was extracted 3, 6, 10, and 30 h after the transfer. As shown in Fig. 2A, it was possible to induce A2 expression in promastigotes with a combination of temperature and pH shifts. Similar results were observed for the *L. donovani* LV9 strain (not shown). Furthermore, A2 gene expression was inducible in amastigote-like organisms of the Sudanese 1S2D strain by a pH shift (Fig. 2B). These amastigote-like organisms cultivated in 100% fetal bovine serum at 37°C (at neutral pH) were shown to share some metabolic and physiologic characteristics

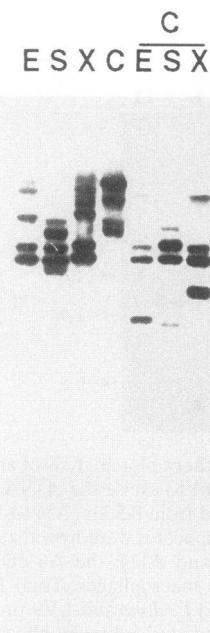


FIG. 3. Genomic organization of the A2 gene family. In each lane, 10 μg of total DNA extracted from *L. donovani* LV9 promastigotes was digested to completion with different restriction endonucleases (E, EcoRI; S, Sall; X, XbaI; and C, ClaI). For double digests, the DNA was first cut to completion with *Cla*I, and the fragments were then precipitated and resuspended in the appropriate buffer for the second digest. Restriction fragments were analyzed by Southern blot analysis with a *Pst*I-*Xho*I fragment of the A2 cDNA insert (0.5 kb).

of intracellular amastigotes (12). On the same panel, this pattern of differential expression was compared with the one obtained with *L. donovani* LV9 promastigotes and amastigotes. Taken together, these data indicate that the pH shift is the major trigger for the developmental expression of the A2 gene but that a combination of pH and temperature shifts is needed for induction of expression.

A2 transcripts are encoded by a multigene family. The genomic organization of the A2 genes was analyzed by Southern blot analyses. Total DNA was digested to completion with several restriction endonucleases, separated on an agarose gel, and hybridized with a probe prepared with the A2 cDNA insert (the A2 cDNA did not contain sites for these restriction enzymes). As shown in Fig. 3, the hybridization pattern in each lane displayed a series of bands of different intensities, clearly showing that many copies of the gene were present in the genome. However, common bands for the EcoRI, XbaI, and Sall digests (between 6 and 10 kb) suggested an arrangement in tandem array. Indeed, if each of these restriction sites is unique in the A2 gene, then copies clustered in tandem array would display fragments of the same length for each digest. In order to verify this assumption and isolate a genomic fragment carrying the complete coding region of the A2 transcript, we isolated and characterized genomic clones identified by the screening of an EcoRI partial genomic library with an A2 cDNA probe. The inserts were mapped with several restriction enzymes and displayed similar patterns. One of these clones, GECO 90, was kept for detailed characterization. Figure 4 (upper panel) shows the restriction map of the fragment and how it corresponds to the A2 cDNA series. It contained unique sites for Sall and XbaI but no *Cla*I site, and this was consistent with the Southern blot analysis shown in Fig. 3. The DNA

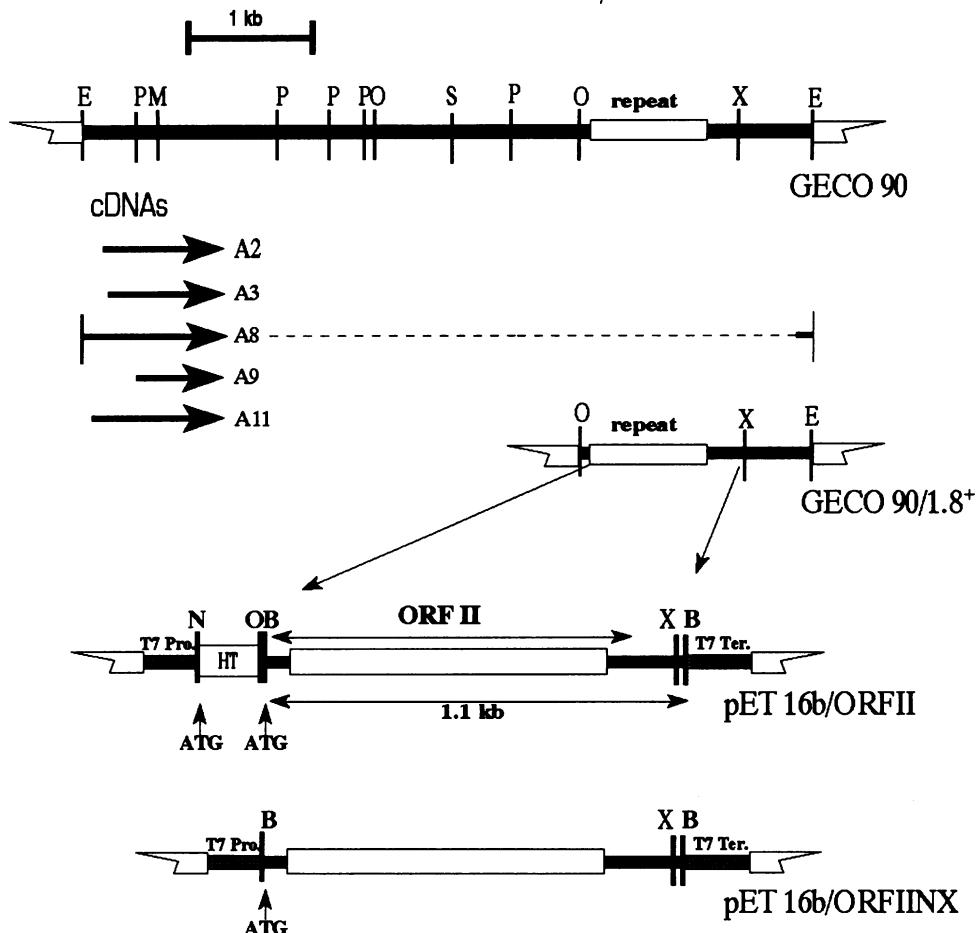


FIG. 4. Restriction map of the genomic clone GECO 90 and corresponding cDNAs (A2 series: upper diagram). The sequence within the *Xba*I-to-*Xba*I fragment which contained the open reading frame (ORF II) and the repeated region (open box) was subcloned into the pET16b vector as shown (lower diagram). Details are provided in the text. B, *Bam*HI; E, *Eco*RI; M, *Sma*I; N, *Nco*I; O, *Xho*I; P, *Pst*I; S, *Sall*; X, *Xba*I; T7 Pro, T7 RNA promoter and termination sequences, respectively. Note that there are two in-frame ATG start codons in the pET16b/ORFII recombinant plasmid; the first gives rise to a fusion protein containing 23 extra amino acids corresponding to a HT. This latter can be cleaved from the fusion protein by proteolytic digestion with factor Xa. The second ATG is part of the subcloned fragment and is the original start codon of the ORF II. In the pET16b/ORFINX construct, the HT was removed from the coding sequence.

sequence flanking each *Eco*RI site on this genomic clone was determined and corresponded to the related portion of the A8 cDNA, confirming that this fragment represented one unit of a tandem array. This genomic clone, therefore, contained the 3' end of an A2 gene and the 5' end of the following unit. Considering that several other hybridization bands were observed for each digest, it is therefore not clear whether all copies of the A2 gene are arranged in tandem arrays or whether more than one cluster exists.

The A2 gene product bears a repetitive unit. The DNA sequence of the 1.9-kb *Xho*I-*Eco*RI fragment of the GECO 90 genomic clone corresponding to the 5' end of the 3.5-kb A2 transcript was determined and compared with the cDNA sequences. The longest open reading frame (ORF II) found was contained in the *Xho*I-*Xba*I 1.1-kb fragment and potentially encoded a 22-kDa protein product (A2 protein). Stop codons were observed in the two other frames and upstream from the initiating ATG. Most of this predicted A2 protein is composed of a repetitive sequence consisting of a stretch of 10 amino acids repeated 19 times (Fig. 5A). Since each unit of this repeat contains two serines, two valines, two leucines, and two prolines separated from each other by five residues, the

repeated region could also be considered as a stretch of 5 amino acids repeated 38 times. The predicted amino acid sequence was compared with proteins listed in Swiss-Prot data bases by using a Fasta algorithm (Canada Institute for Scientific and Technical Information; Scientific Numeric Databases Service). The most striking identity was observed with an S antigen of *P. falciparum*. As with the *L. donovani* A2 protein, the carboxy-terminal portion of this S antigen of *P. falciparum* Vietnamese isolate VI is composed of a stretch of 11 amino acids repeated 19 times (7); the repeated units of both proteins are 50% identical and 80% homologous (Fig. 5B).

It was important to verify whether the A2 protein derived from the coding region was indeed expressed by the *L. donovani* parasite. Therefore, we tested the possibility that the A2 protein was recognized by kala-azar immune sera. To produce the protein, the coding region from the initiating ATG to the *Xba*I restriction site was subcloned in the pET 16b expression vector in frame with the HT encoded by the vector (Fig. 4, lower diagram). In an immunoprecipitation assay, the A2 fusion protein of 27 kDa produced in an in vitro transcription-translation assay was recognized by the kala-azar immune serum (L1 [Fig. 6A]). This serum did not react against the

A**Xba I**
GAGCTCCCCCAGCGACCCTCTCGCAACCGAGCGCCCCAGTCCCCCACGCACAACTTGACCGAGCACA**ORF II→**

Met Lys Ile Arg Ser Val Arg Pro Leu Val Val Leu Val Cys Val Ala Ala Val Leu Ala Leu
 1 ATG AAG ATC CGC AGC GTG CGT CCG CTT GTG GTG TTG CTG GTG TGC GTC GCG GCG GTG CTC GCA CTC
 Ser Ala Ser Ala Glu Pro His Lys Ala Ala Val Asp
 67 AGC GCC TCC GCT GAG CCG CAC AAG GCG GCC GTT GAC

Val Gly Pro Leu Ser Val Gly Pro
 103 GTC GGC CCG CTC TCC GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 127 CAG TCC GTC GGC CCG CTC TCT GTT GGC CCG
 Gln **Ala** Val Gly Pro Leu Ser Val Gly Pro
 157 CAG GCT GTT GGC CCG CTC TCC GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 187 CAG TCC GTC GGC CCG CTC TCT GTT GGC CCG
 Gln **Ala** Val Gly Pro Leu Ser Val Gly Pro
 217 CAG GCT GTT GGC CCG CTC TCT GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 247 CAG TCC GTT GGC CCG CTC TCC GTT GGC CCG CTC TCC GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly **Ser**
 292 CAG TCT GTT GGC CCG CTC TCC GTT GGC TCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 322 CAG TCC GTC GGC CCG CTC TCT GTT GGT CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 352 CAG TCC GTC GGC CCG CTC TCC GTT GGC CCG
 Gln **Ala** Val Gly Pro Leu Ser Val Gly Pro
 382 CAG GCT GTT GGC CCG CTC TCC GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 412 CAG TCC GTC GGC CCG CTC TCT GTT GGC CCG
 Gln **Ala** Val Gly Pro Leu Ser Val Gly Pro
 442 CAG GCT GTT GGC CCG CTC TCT GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 472 CAG TCC GTT GGC CCG CTC TCC GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 502 CAG TCT GTT GGC CCG CTC TCC GTT GGC TCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 532 CAG TCC GTC GGC CCG CTC TCT GTT GGT CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 562 CAG TCC GTC GGC CCG CTC TCC GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 592 CAG TCT GTC GGC CCG CTC TCC GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 622 CAG TCC GTC GGC CCG CTC TCC GTT GGT CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 652 CAG TCC GTT GGC CCG CTC TCC GTT GGC CCG
 Gln Ser Val Gly Pro Leu Ser Val Gly Pro
 682 CAG TCC GTT

Asp Val Ser Pro Val Ser ***
 691 GAC GTT TCT CCG GTG TCT TAA GGCTCGCGTCCGTTCCGGTGTGCGTAAAGTATATGCCATGAGGCATGGTGACGAG
 771 GCAAACCTTGTCAAGCAATTGTCGCTTACCGTCAAGAGCACACAGCAGACTGAGTGTTCAGGTGGCACAGCACACGCT
 857 CCTGTGACACTCCGTGGGTGTGTGACCTTGCTGCTGTTGCCCAGCGGATGA
 943 TTCCAACCTTGCACCTTCACGCCACAGACGCATAGCAGGCCCTGCTGCGGCCATGCGGGCAAGCCATCTAGATGCGCCT
 Xba I
 1029 CTCCACGACATGGCGGAGGGCGCAGATGAAGGCAGCGACCCCTTTCCCGGCCACGACGCCGCTGAGGGGGCCCCACAGCG
 1115 CAGAACCTGCGAGCGCGTGCAGGGCGCTGTGACGCACAGCCGCACGCAGCGTACCGCACGCAGACAGTCATGGGAGGGCGGA
 1201 GGAGCAAGAGCGGTGGACGGAACCGCGCAAGCATGCGGCACGCCCTCGATGTGCTGTGGCTGATGAGGCGGGATGCCGG
 1287 AAGCGTGGCGAGGGCATCCGAGTTGACCGTCGAGTCTCCAGGCCAATGTGGCAGCCTGCGGGGAGCAGATTATGGGATGC
 1373 GGCTGCTCGAACGACCGAGGGCGCTGACCGGAAGGTGGCCACTTCCTCCGGCTGTGCGGCATCCGCCCTCGATCGGAGC
 1459 CCGAACATGGTGGCCCGCGGGTGAAGGCGTCCGCCACCCCGTCCCGTGTGGCGCCCTGGGGCAGGTGCGCTGTGGCTGTGT
 1545 ATGTGCGCTGATGTGCTGACTTGTGCTGGCTATGGCACGGTGAGGGCGACGTTGGCCCTGCTGACTTCCTCTGCTTTC
 1631 TTATTATTCTCAGTGGCCCCCGCTGGATTGGCTGCATGGCGCTGTGATCGCCTGCTCTCTCATTGACGGCTGCGGCCCTC
 Eco RI
 1717 CCGCCCTCCCACTGTGCTGTGGATGGAGGCACGGCGGGCTCTGTGTTGTCACCGCGTCAAGAATTAGATGAGGGACT
 1803 ^{A8→}CCCGAGCGAGCACAAAGCAGCAGCACAGGAGGCAGGCCCTGAGCACGTTTCTTCTCTTCTGAGACTGCGGACTACGG
 1889 GAATCAGAGACGTCGTCAGAGACGCGCATCCGACCCCGCGCTATGCTTCTCGTCTCTCCGCCCTTCTCTTCTGCAAGCGCTT
 1975 CTGTCGCGTGTGCGAGCGCCGTTGCCGGCTCTCTCCCTCCCTCTCTCTGCAAGCGCTTCTTACAGGCC
 2061 GAACGTTGCTGCTCGCCTGGAGGCCGTTCCCTCTTATCATCTGCAATTATTACACGTCGCTTGTGCTTGTGCTTCTGAC
 2147 GATGCCGCCACCTCACCGGGTGTCAAGGCCAGCGCCCACCTCTTGTGGCAGGCCAGTACGCTGCAGCCTGCCATGAGCAC
 2233 GGCTGTTGACTCTTGGTGCAGCGGACAGGTGTGGCTGGCGCTGCGCTGACACCAACGGTATGACGCTTGACCGAC
 2319 CACTGCCGATCATGCCGACGATTCAACGAATGCGCGCATCCACTGCGCTTCTGCGCTGCGCTGCCGGTGTGAGCG
 Sma I
 2405 TGGTCCGGGCCCTAGCGCGCTGTACCGAGCGGATTGCGTGGCTGAGCGGCCAGGCCAGGGTGTGGCCGGCTGCTGGC
 2491 GGCATAGCGTGGCGTGCAGCAGATGCGGATGGGCTGCGCTGCGCATGCGTGTGCGTTGACTTGTGCGTTGGCGGGCAGC
 2577 TAAACGGAAAATGCGCTTGGCTTCCCGGCCACGCTCCGGCGCTGGTGGCTATTGCAATCGCGCTGAAGAGGTGGCGAGG
 2663 AAAATGGCACGAGGCCAGAGGGAAAAACGAAAAGTGCACAAAGTGCACAAACGCCAGAAAATGCGGGAAAAGCAGAAAAGTGCA..
 A2, 3, 8, 11 J A9 J

B
A2 10 20 30 40 50
 MKIRSVRPLVVLLCVAAVLALSASAEPHKAAVDVGPQLSVPQSV-GPLSVE
 : : : : : | | | | | : | | | |
Sant_P PGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEG
 110 120 130 140 150 160
 60 70 80 90 100
A2 PQAV-GPLSVPQSV-GPLSVPQAV-GPLSVPQSVGPLSVPQSV-GPLSVE
 | : : | | | : : | | | | : : | | | | : : | | | | : | | | : |
Sant_P PKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTG---GPGSEGPKGTGGPGSEG
 170 180 190 200 210 220
 110 120 130 140 150 160
A2 QSV-GPLSVPQSV-GPLSVPQAV-GPLSVPQSV-GPLSVPQAV-GPLSVPQSV-G
 : : : | | | : : | | | | : : | | | | : : | | | | : : | | | : |
Sant_P KGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGGPGSEGPKGTGG
 230 240 250 260 270 280
 170 180 190 200 210
A2 PLSVPQSV-GPLSVPQSQSV-GPLSVPQSVGPLSVPQSVGPLSVPQSVGPLSVPQSV
 | | : : : | | | | : : : | | | | : : | : : : | | : : | : : : | : : : |
Sant_P PGSESPKGTTGGPGSEGPKGTGGPGSEGPKGTGGPGSEAGTTEGPKGTTGGPGSEAGT
 290 300 310 320 330 340
 220 230
A2 VGPLSVPQSVDPVSPVS
 | | : : |
Sant_P EGPKGTGGPGSGGEHSHNKKSKKSIMNMLIGV
 350 360 370

FIG. 5. (A) Partial nucleotide sequence of the A2 gene carrying the ORF II coding region and deduced A2 protein sequence. The sequence from the *Xba*I site to the beginning of cDNAs was determined from the genomic clone GECO 90 and connected to the A2 cDNA sequence. The A of the initiation codon ATG was given number +1. Boldface nucleotides mark the 5' ends of the A2 series cDNAs corresponding to this sequence. Underlined nucleotides at the end of the sequence mark the polyadenylation sites; the A9 cDNA contains extra bases at its 3' end. (B) Alignment of A2 protein sequence (A2) with the amino-terminal portion of the S antigen of *P. falciparum* isolate VI (Sant_P). Dashes are for identical residues and dots are for homologous amino acids.

control protein product, human p53. To confirm the specificity of the immune reaction, the pET16b/ORFII plasmid coding for the recombinant A2 fusion (HT-A2) protein was expressed in *E. coli*, and the recombinant protein was tested for reactivity with the L1 serum by Western blot analysis. As shown in Fig. 6B, the kala-azar serum reacted specifically with protein products of 27.5 and 25 kDa in the lysates derived from cells containing the pET16b/ORFII plasmid. The 25-kDa protein probably corresponded to the A2 protein without the HT; the sequence subcloned contained the original ATG of the A2 predicted protein (Fig. 4). The serum did not react against proteins from *E. coli* lysates carrying the pET16b plasmid without the insert. These data argue that the ORFII encodes for an *L. donovani* protein and that this product was antigenic in this patient with visceral leishmaniasis.

Processing of the A2 protein. When compared with protein sequences in data bases, the amino-terminal portion of the predicted A2 protein showed significant homologies with leader sequences of unrelated proteins. This sequence (from amino acids 1 to 32) possess the characteristic tripartite domain structure of eukaryotic signal peptides: a positively charged *n* region consisting of the first eight amino acid residues, a hydrophobic stretch (*h* region; extending to position 22), and the *c* region containing polar amino acids (26). We, therefore, tested the possibility that the putative signal peptide sequence could be processed *in vitro* by microsomal vesicles. In these assays, the HT portion was removed from the pET16b/ ORFII recombinant plasmid to allow only initiation of translation from the original ATG of the A2 protein (pET16b/ ORFIINX [Fig. 4]). Figure 7A shows the proteins produced by

each of these two plasmids when used as templates in *in vitro* transcription-translation assays. When CPMM were included in the reaction, the A2 protein product labelled with [¹⁴C]leucine was detected in the microsomal fraction as a lower-molecular-weight product (Fig. 7B). The latter could not be detected if [³⁵S]cysteine was used in the assay, confirming that the only cysteine residue present in the protein (position 15) was part of the cleaved signal sequence. These results confirmed the presence of a functional signal peptide at the amino-terminal end of the A2 protein, which could be recognized by signal recognition particles and used for translocation. These data are consistent with the A2 protein being secreted from *L. donovani* amastigotes.

DISCUSSION

Leishmania protozoans must undergo several metabolic and physiological changes throughout their life cycle. Understanding the mechanisms governing the developmental expression of stage-specific products essential for adaptation in each environment will contribute to defining the molecular basis for the infectivity and the pathogenicity of the parasite. In the present study, we describe the identification and characterization of the A2 gene which is highly expressed in amastigote-infected macrophages. The induction of expression of the gene in promastigotes required a combination of temperature and pH shifts, conditions which mimic the passage from the insect to the mammalian host. The A2 protein product is similar in sequence and structure to a developmentally expressed S

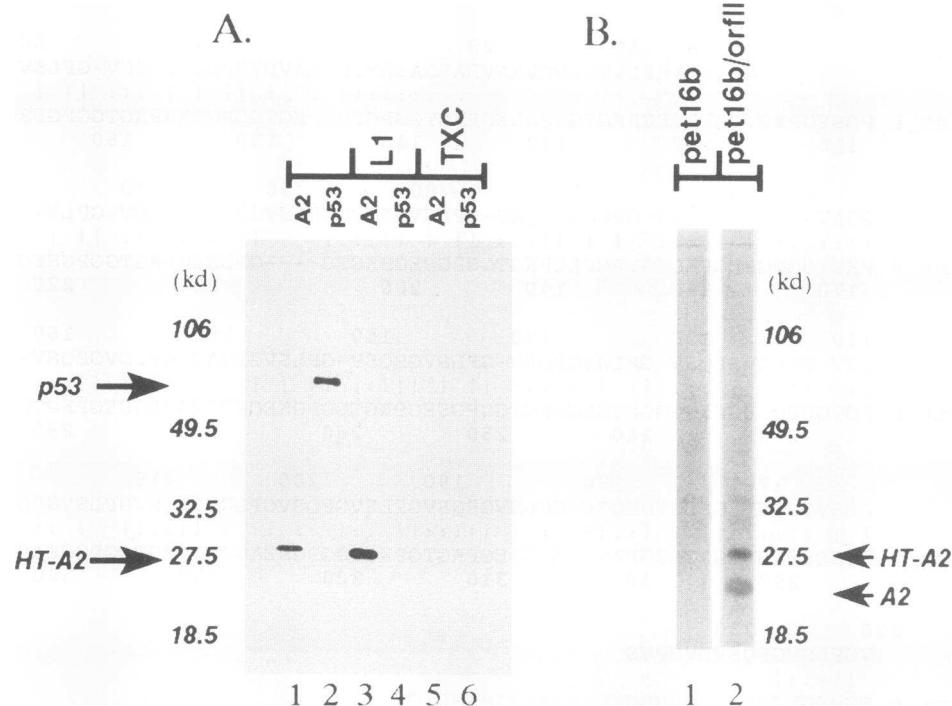


FIG. 6. Immunoprecipitation and Western blot analysis of the A2 protein with sera from a patient with kala-azar. (A) The in vitro-translated HT-A2³⁵S-labelled fusion protein was immunoprecipitated with kala-azar immune serum and analyzed by SDS-polyacrylamide gel electrophoresis. Labelled proteins were synthesized by an in vitro transcription-translation assay with the pET16b/ORFII plasmid and a control pBluescript/p53 plasmid as templates. Lanes 1 and 2, labelled protein products prior to immunoprecipitation analysis. Lanes 3 and 4, immunoprecipitated proteins with the kala-azar immune serum (L1). Lanes 5 and 6, immunoprecipitated protein with a control human serum (TXC). (B) Western blot analysis of the A2 recombinant protein synthesized in *E. coli*. Lanes 1 and 2, reaction of kala-azar immune serum against total protein from isopropyl-β-D-thiogalactopyranoside-induced *E. coli* DE3 cells carrying the control pET16b plasmid or the pET16b/ORFII recombinant plasmid, respectively. The kala-azar immune serum was from a young Iranian patient suffering from visceral leishmaniasis and reacted strongly against *L. donovani* antigens in an enzyme-linked immunosorbent assay (37a).

antigen of *P. falciparum*, suggesting that these two proteins carry out similar functions during infection.

Several investigators have focused on the identification of stage-specific products of *Leishmania major*, the causative agent of cutaneous leishmaniasis. Coulson and Smith (9) isolated cDNAs representing transcripts mostly present in both metacyclic promastigotes and amastigotes. One of these cDNAs has been shown to encode a HSP 70-related product. Brodin et al. (6) described the isolation of a full-length cDNA developmentally expressed in metacyclic promastigotes; the anticipated product contained a B-ZIP motif and could encode a regulatory protein. Other studies have characterized heat-inducible products in different strains of the *Leishmania* parasite (16, 18, 35–37, 40). Most recently, two genes that have been identified by the differential screening of an *L. donovani* axenic amastigote cDNA library were shown to be expressed twofold higher in amastigotes than in promastigotes (15). In contrast to the above previous studies, this is the first report describing the isolation and characterization of a gene, other than heat shock genes, which is highly expressed in amastigotes and only weakly expressed in promastigotes. The A2 gene system, therefore, represents an interesting model system to investigate the regulation of gene expression in *Leishmania* cells.

The differential screening of an amastigote cDNA library with stage-specific probes allowed us to identify seven cDNA clones representing transcripts expressed only in the amastigote stage. Five of these were shown to represent the same or

closely related genes (A2 series). In practice, the differential hybridization screening method allows only the identification of transcripts which are relatively abundant in one cell type. However, considering the obvious morphological and physiological differences between both forms of the *Leishmania* parasite, the number of clones found appeared low. This may suggest that there are relatively few highly expressed amastigote-specific genes. It is also noteworthy that there were many more easily identifiable cDNA clones representing promastigote up-regulated transcripts than amastigote-stage-specific transcripts, and this was despite the fact that the cDNA library was constructed from amastigote mRNAs. This suggests a down-regulation of expression of numerous genes during the cytodifferentiation from promastigote to amastigote.

The 3.5-kb amastigote-specific transcript recognized by the A2 cDNA series was induced to accumulate in promastigotes following temperature and pH shifts; a temperature shift by itself was not sufficient. A maximal level of expression of the A2 genes was observed several hours after the transfer at 37°C and pH 4.5, and this long lag time suggests that the developmental expression was not a direct response to physicochemical changes but rather a subsequent event in the cytodifferentiation process. We confirmed that the A2 genes were also expressed in amastigote-infected macrophages and in amastigotes extracted from the spleens of infected animals. The A2 gene is not expressed in amastigote-like organisms when cultured at neutral pH (37°C), clearly showing that pH, as much as temperature, is an important trigger for promastigote-

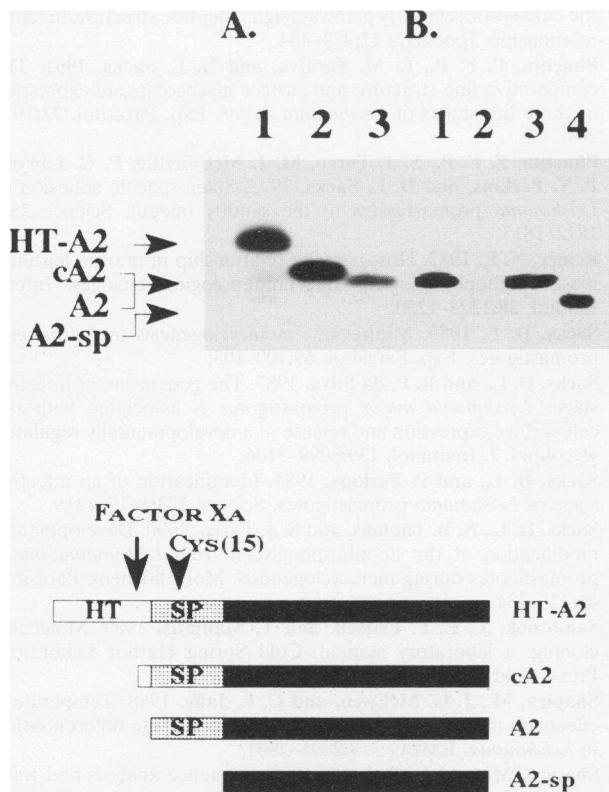


FIG. 7. In vitro processing of the A2 protein. (A) [35 S]methionine-labelled protein produced in in vitro transcription-translation assays with the recombinant plasmids pET16b/ORFII (lane 1 and 2) or pET16b/ORFIINX (lane 3) as templates. In lane 2, the HT was removed from the HT-A2 fusion protein (27.5 kDa) by proteolytic digestion with factor Xa protease. The resulting product (cA2; 25 kDa) contains six extra residues at the amino-terminal end compared with the A2 protein (lane 3). (B) CPMM processing of the A2 protein labelled with [35 S]cysteine (lanes 1 and 2) or [14 C]leucine (lanes 3 and 4). Lanes 2 and 4, purified microsomal fraction samples, the cleaved product is about 23 kDa (A2-sp). Lanes 1 and 3, TNT assay reaction samples without CPMM, as controls. Details are provided in the text. For both panels, samples were analyzed by SDS-polyacrylamide gel electrophoresis (12%). Gels were treated with Amplify reagent (Amersham), dried, and autoradiographed for 18 to 96 h.

to-amastigote cytodifferentiation. Previous studies emphasized the importance of pH for the transformation of the parasite and its maintenance as an amastigote. In particular, Zilberstein et al. showed that specific antigens were expressed in promastigotes following their transfer into low-pH medium (42). Bates et al. (3) also demonstrated that *L. mexicana* amastigote-like cultures, initiated from lesion-purified amastigotes, require an acidic environment to conserve their characteristic round amastigote morphology. However, since the amastigotes used in our experiments were incubated for 18 h at neutral pH prior to the RNA extractions, it argues that while pH shift was necessary as a trigger, it was not required for maintenance of the A2 expression.

The A2 transcript-encoded protein contains a repetitive sequence which has identity with the repeated unit of an S antigen of *P. falciparum*, the causative agent of malaria. S-antigen genes are differentially expressed by mature merozoites in their parasitophorous vacuoles and form a fuzzy layer around the parasite; as the erythrocyte bursts, the S antigens are released into the serum. It has been suggested that the S

antigens, having repeated epitopes, could act as circulating antigen absorbants (smoke screen effect) (1, 8, 10). In a similar manner, the A2 protein carrying a functional signal peptide sequence could also be excreted out of the cell and accumulate in the phagolysosome until the macrophage cell bursts. Released antigens might then prevent the opsonization of the parasite until it is phagocytosed by another macrophage. We are now undertaking to produce antibodies against the A2 protein and the repeated section in order to characterize its pattern of expression in the parasite and in infected cells during the cytodifferentiation.

Recently, Birse et al. (4) reported the identification of a developmentally regulated transcript expressed in association with hypha formation in *Candida albicans*. The hyphal form allows the pathogen to invade tissues, and this transformation represents an important step in the infection process. Similar to the A2 gene, the expression of the ECE1 (extended cell elongation 1) gene was pH and temperature dependent, and most of the ECE1 protein is composed of a repeated section.

Development of novel techniques and methodologies allowing homologous recombinations in *Leishmania* cells opens up a variety of possibilities for analysis of the biological importance of the A2 genes in amastigotes. This could also provide insights into the biological role of developmentally expressed antigens bearing repetitive sequences during infection with human pathogens.

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Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against *Leishmania donovani* infections

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Abstract

The A2 genes of *Leishmania donovani* encode amastigote-specific A2 proteins, which are considered to be virulence factors required for the survival of this protozoan parasite in the mammalian host. The A2 genes are present within a multigene family and corresponding A2 proteins are composed predominantly of multiple copies of a 10 amino acid repeat sequences. A2-specific antibodies have been detected in the sera of patients suffering from visceral leishmaniasis (VL) and it has been shown that generation of A2 deficient *L. donovani* resulted in an avirulent phenotype. In this report, we show that immunization of mice with recombinant A2 protein conferred significant protection against challenge infection with *L. donovani*. The protection correlated with in vitro splenocyte proliferation, production of IFN- γ in response to A2 protein and the presence of A2-specific antibodies in the sera of immunized mice. These data demonstrate that A2 represents a potential antigen for protection against infection with *L. donovani* and VL. © 2001 Published by Elsevier Science Ltd.

Keywords: Vaccine; *Leishmania donovani*; Macrophage

1. Introduction

Leishmaniasis is a spectrum of diseases caused by infection with different species of the protozoan parasite *Leishmania* [1–4]. These diseases range from self-limiting cutaneous leishmaniasis (CL) to visceral leishmaniasis (VL), also known as Kala-azar, which is a fatal infection if not treated successfully. Leishmaniasis effects over 12 million people in 88 countries, over 350 million are at risk, and over 2 million new cases emerge every year [1–3]. Different species of sandfly transmit *Leishmania* and reservoirs include canine, wild rodents, and human. Within the insect host, *Leishmania* is present as flagellated promastigote form and upon infecting the mammalian host it differentiates into the smaller aflagellated round amastigote stage and multiplies in the phagolysosome vacuole of macrophages. Leishmaniasis is difficult to treat and there is increasing resistance developing against the currently available drugs [5]. New disease foci are identified every year in different parts of the world and this may be due to the emerging resistance of sandflies towards insecticides [6] and resistance of the parasite to the existing chemotherapy. In developing and underdeveloped parts of the world, acquired immunosuppressive syndromes (including AIDS) add to the

higher risk of leishmaniasis [7]. Based on these and other observations, there is clearly an urgent need for vaccine development against this disease and in particular against fatal Kala-azar.

Several vaccine clinical trials against CL have been undertaken [8–10] and more recently a trial has been carried out against VL in the Sudan [11]. Most experimental vaccines against leishmaniasis have been either live strains [12–14], defined subunit vaccines [15–18] or crude fractions of the parasite [19]. Recently, DNA vaccines have resulted in protection in experimental CL and have appeared to preferentially induce a Th1 immune response [20]. There are considerably more protective antigens described for *L. major* infections (example [21–23]) as compared to *Leishmania donovani* in this respect [24–27]. All of the experimental vaccine candidates for *L. donovani* are thought to produce T-cell mediated responses and high antigen-specific antibody titres [24,25,27]. Since few experimental vaccination studies have been carried out against VL, we have therefore examined the possibility of using the A2 antigen of *L. donovani* as a vaccine candidate against VL.

A2 genes are expressed specifically in *L. donovani* amastigotes [28]. The A2 genes are present within a multigene family and the corresponding A2 proteins are composed of predominantly multiple copies of a 10 amino acid repeat sequence. The A2 proteins are abundant in amastigotes and range in molecular weight from 42 to 100 kDa

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depending on the number of repeats within each protein species [29,30]. A2-specific antibodies have been identified in the sera of patients suffering from VL [31]. It has been shown that generation of A2 deficient *L. donovani* resulted in avirulent amastigotes, which could not survive in a mouse model but were able to multiply in axenic culture as promastigotes [32]. All these above findings tempted us to investigate the potential of using the A2 antigen as a vaccine candidate against *L. donovani* infection.

2. Materials and methods

2.1. Leishmania strain and mice

L. donovani Sudanese 1S2D promastigotes and amastigotes were cultured as described previously [32]. Female BALB/c (Lsh^s, H-2^d) and C57B/6 mice (4–6 weeks old) were obtained from Charles River, Canada.

2.2. A2 immunization and challenge infection

A2 was purified from *E. coli* BL-21 containing pET16bA2 plasmid. Endotoxin free recombinant A2 protein was used for vaccination and other studies. Mice were injected i.p. with A2 protein combined with 100 µg heat killed *Propianibactrium acnes* (Elkins. Sinn, Cherry Hill, NJ) as the adjuvant for the first injection and subsequent boosts were with A2 protein in PBS in the absence of adjuvants. For the vaccination studies, the antibody response experiments, and for passive immunization studies, each mouse received 10 µg of recombinant A2 protein for the first injection and 5 µg each for the two boosts with 3-week intervals between each injection. Control mice received only 100 µg heat killed *P. acnes* as the adjuvant for the first injection and subsequent boosts were with PBS. Mice were bled 3 weeks following the final injections and serum from the mice in each group ($n = 4$) were pooled. For the vaccination experiment, mice were immunized as above and then challenged 3 weeks after the final boost and euthanized for liver biopsies 4 weeks following challenge. For challenge infection, 2×10^8 stationary phase cultured promastigotes of *L. donovani* (1S2D) were injected in the tail vein in 100 µl PBS per mice. For passive immunization, 3 weeks after the final boost 8×10^8 splenocytes were collected and transferred to naive mice by tail iv. One week after the transfer mice were challenged with 2×10^8 *L. donovani* promastigotes and 4 weeks after the challenge infection mice were killed and parasite burden were measured by liver touch biopsy.

For the cell proliferation and cytokine production assays, mice were immunized with 10 µg recombinant A2 protein and 100 µg heat killed *P. acnes* in the first injection and 5 µg of A2 protein in PBS for 1 boost injection at 2-week intervals. Control mice received only 100 µg heat killed *P.*

acnes for the first injection and the subsequent boost was with PBS. Two weeks after the boost, mice were euthanized and spleens were isolated. Spleens from mice in the same group (four per group) were pooled together.

2.3. Vaccination analysis

Four weeks following challenge infection, mice were euthanized and liver touch biopsies were microscopically examined after fixing and staining the slides with Giemsa [33]. Leishman donovan unit (LDU) were calculated as LDU = (number of amastigotes/number of liver nuclei) × weight of liver in milligrams [34]. Protection studies were performed in 4 mice per group and the experiment was repeated three times with similar results.

2.4. ELISA

The method for end point titration was described elsewhere [35]. In brief, 5 ng of recombinant A2 protein was coated per well in a 96 well plate at 4°C overnight in 50 µl binding buffer (0.1 M NaPO₄, pH 9.0). The wells were washed three times with PBS-T (PBS, 0.1% Tween 20) and blocked with 200 µl of 3% bovine serum albumin (BSA) in PBS-T for 2 h at 37°C. Wells were then washed three times with PBS-T and incubated with 100 µl of diluted serum (serially diluted at 2-fold starting at 1:20 in PBS-T, 1% BSA) for 2 h at 37°C. Wells were then washed three times with PBS-T and incubated with 1:2000 diluted HRPO conjugated anti-mice goat antibody in PBS-T, 1% BSA for 1 h at 37°C. After washing three times with PBS-T, the color was developed with TMB-ELISA (Life Technology) as manufacturers recommendation. The cut-off was determined as: 3 × (mean blank OD + S.D. of blanks).

For cytokine capture ELISA of IL-4 and IFN-γ, 5 × 10⁶ per single spleen cell suspensions in RPMI-1640 were stimulated with 50 ng/ml recombinant A2 antigen and culture supernatant were collected after 96 h. The concentration of IFN-γ and IL-4 in the resulting supernatant was determined as described previously [36] using biotinylated capture antibody followed by streptavidin conjugated to HRPO (Pharmingen).

Isotype-specific antibodies were purchased from Sigma and antigen mediated ELISA were performed according to suppliers instructions. In brief, 100 ng of recombinant A2 protein in 100 µl were coated over night at 4°C in 0.1 M phosphate buffer pH 9.0 and blocked with 200 µl of 3% BSA in PBST for 1 h at room temperature and washed three times with PBST. Mouse sera (100 µl) diluted to 1:100 in PBST was added to the wells and incubated at room temperature for 2 h then washed three times with PBST. Goat anti-mice isotype antibodies were incubated at 1:1000 dilution for 1 h washed again and rabbit anti-goat-HRPO at 1:5000 dilution was incubated for 0.5 h and the color was developed with TMB-ELISA. All samples were run in triplicates.

2.5. Cell proliferation assay

Single cell suspensions of isolated splenocytes (4×10^6 cells/ml) were stimulated with 0.5 µg/ml of recombinant A2 in 200 µl in a 96 well plate at 37°C, 5% CO₂ for 72 h and pulsed for additional 18 h with 1 µCi of [³H] thymidine per well. The plate was harvested and the amount of incorporated [³H] thymidine was measured in a β-counter. Results are represented as the difference in counts obtained between the A2-stimulated and non-stimulated controls.

2.6. Western blot analysis of A2

The SDS-PAGE (12%) was run with 1 µg of recombinant A2 protein in each lane. The resolved proteins were then transferred to a nitrocellulose filter in the presence of 20% v/v methanol, 25 mM Tris, pH 8.2, 190 mM glycine at 30 V for 12 h. Filters were washed then incubated directly in anti-A2 C9 hybridoma supernatant [30] with 5% milk in PBS-T for 2 h at 22°C then washed and incubated in the presence of horse radish peroxidase labeled anti-mouse IgG in PBS-T at room temperature for 1 h. The membrane was then incubated in Amersham ECL detection solution for 1 min and then exposed to X-ray film followed by autoradiography.

2.7. Infection of macrophages with amastigotes

Bone marrow derived macrophages (BMMs) were obtained from femurs of 6–8-week-old female BALB/c mice as previously described [19]. Quiescent BMM (10^6 cells/ml) were infected with cultured amastigotes at a ratio of 1:1 amastigote per macrophage for 24 h in polystyrene tubes. The infected BMMs were washed extensively for four times with 50 volume PBS at 900 rpm for 10 min. Internalization of parasites was measured by microscopic count of Giemsa-stained cytocentrifuged slides. The sera were de-complemented by incubating at 65°C for 2 h in a water bath.

2.8. Statistical analysis

Significance of difference was examined by Student's *t*-test using "GraphPad PRISM" (version 3.02) software with 99% confidence intervals and a value of $P < 0.05$ was considered statistically significant. The *P*-values and S.E. values reported were determined from the replicate measurements (minimum 3) within each experiment. Each experiment was repeated two or more times with similar outcomes.

3. Results

3.1. Immunization with A2 protein protects mice from *L. donovani* infection

We initially determined whether immunization with the recombinant A2 protein was protective against in-

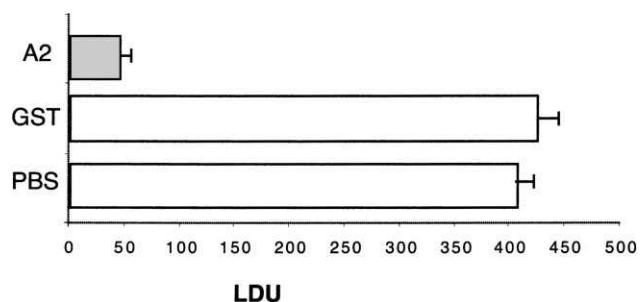


Fig. 1. Infection levels following A2 protein vaccination as determined by LDU. BALB/c mice were immunized with recombinant A2 or recombinant GST protein three times at 3-week intervals as described in Section 2. Three weeks following the final injection, the mice were challenged i.v. with 2×10^8 *L. donovani* promastigotes. Four weeks after the challenge infection, mice were killed and LDU was calculated from liver biopsies. The mean LDU ± S.E. is shown ($n = 4$ mice per group). This result is the representative of three independent experiments.

fection from *L. donovani* in BALB/c mice. As described in the introduction, the A2 protein is a *L. donovani* amastigote-specific gene product which is highly expressed in infected macrophages. Mice were immunized with recombinant A2 protein as described in Section 2 and 3 weeks after the final injection; BALB/c mice were challenged with *L. donovani* promastigotes. The degree of protection against infection was evaluated by amastigote levels in the liver touch biopsies represented as LDU. As shown in Fig. 1, A2 protein immunization had reduced the LDU by 89% over the control mice or recombinant GST protein-immunized mice ($P < 0.0001$). These data demonstrate that vaccination with the recombinant A2 antigen provided a significant level of protection against infection.

3.2. High-specific antibody titer generated in mice immunized with A2

The above observations demonstrated that the recombinant A2 protein immunization provided a significant level of protection against infection. It was therefore necessary to characterize the immune response generated against the A2 antigen. To determine the titer of anti-A2 antibodies in each immunized group of mice, an ELISA end point titration was performed. As shown in Fig. 2A, the antibody response against A2 was much higher in the mice immunized with A2 antigen with a reciprocal end point titre reaching 2560 as compared to mice immunized with adjuvant only.

To confirm that the antibody response was generated against A2, the sera (1:500 dilution) were also tested by Western blot analysis against recombinant A2 protein. As shown in the Fig. 2B, the sera from the mice immunized with recombinant A2 protein demonstrated a specific anti-A2 antibody response. These Western blot data confirmed the ELISA results in demonstrating that A2 vaccination did generate a strong anti-A2 antibody response.

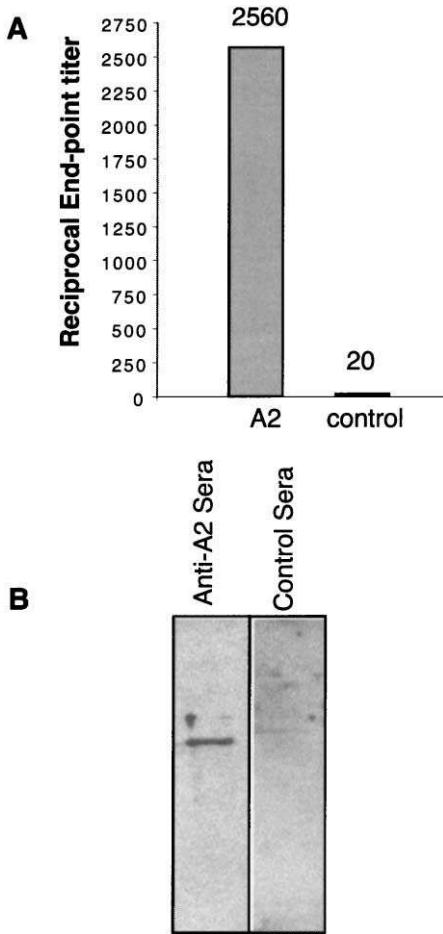


Fig. 2. Relative anti-A2 antibody levels in mice following A2 protein vaccination. Panel A, BALB/c mice were immunized as described in Fig. 1 and anti-A2 antibody levels determined by reciprocal end point titer. This result is the representative of two independent experiments and triplicates were used for each sample. Panel B, western blot analysis of serum for specificity against A2 protein. Serum were used at 1:500 dilution on 1 μ g of recombinant A2 protein per lane.

3.3. Antigen-specific splenocyte proliferation in the mice immunized with recombinant A2 antigen

We next examined the lymphocyte proliferation response to A2 antigen in a mixed splenocyte reaction as described in Section 2. Lymphocytes from a mixed splenocyte preparation were stimulated with recombinant A2 protein in vitro and thymidine incorporation measured. As shown in Fig. 3, thymidine uptake was much higher in splenocytes collected from mice vaccinated with the recombinant A2 antigen. Immunization with the adjuvant alone or PBS resulted in minimal splenocyte proliferation in response to stimulation with A2 protein. Thymidine incorporation was also negligible over background in the former groups when stimulated with an irrelevant recombinant GST antigen (data not shown).

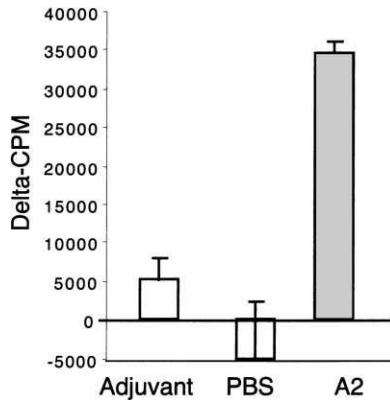


Fig. 3. Proliferation response of splenocytes from mice receiving A2 protein immunization. Mice were immunized with A2 and spleens were collected following the final immunization. Splenocytes were stimulated with recombinant A2 and thymidine incorporation was measured. Delta CPM represents the difference in counts compared with the corresponding non-stimulated cells. Control mice received either adjuvant or PBS.

3.4. Induction of IFN- γ production in response to A2 protein stimulation in splenocytes of immunized mice

It has been established that protection against *L. donovani* infection requires an IFN- γ -activated immune response generated against the parasite [37,38] and production of IFN- γ rather than IL-4 determines the degree of resistance of *L. donovani* infection [39]. We therefore determined whether immunization with the recombinant A2 protein resulted in increased IFN- γ or IL-4 production in response to A2 challenge. As demonstrated in Fig. 4A, splenocytes from mice vaccinated with A2 secreted significantly higher level of IFN- γ ($P < 0.0001$) when stimulated with A2 than splenocytes collected from control mice. Moreover, the release of IL-4 was not significantly higher in the recombinant A2 antigen-immunized mice than control mice following stimulation with A2.

It has been previously shown that IFN- γ production, a marker of Th1 cellular response, directly correlates with a higher IgG2a antibody subclass against the antigen [40], whereas IL-4, a Th2 marker, is associated with generation of IgG1 [41]. We therefore investigated the A2 antigen-specific IgG subclass antibody levels in immunized mice as described in Section 2. As shown in Fig. 4B, all of the A2 antigen-specific IgG subclass titres were significantly higher in mice immunized with recombinant A2 protein than in the control group. These data argue that A2 immunization resulted in stimulating both Th1 and Th2 response against the A2 protein.

Taken together, the A2 antigen immunization data show that the A2 is protective against *L. donovani* infection and was able to stimulate both an antibody response as well as induce IFN- γ production in response to recombinant A2 protein. These data strongly argue that the A2 antigen has the prerequisite characteristics for delivering a protective immune response against *L. donovani* infection.

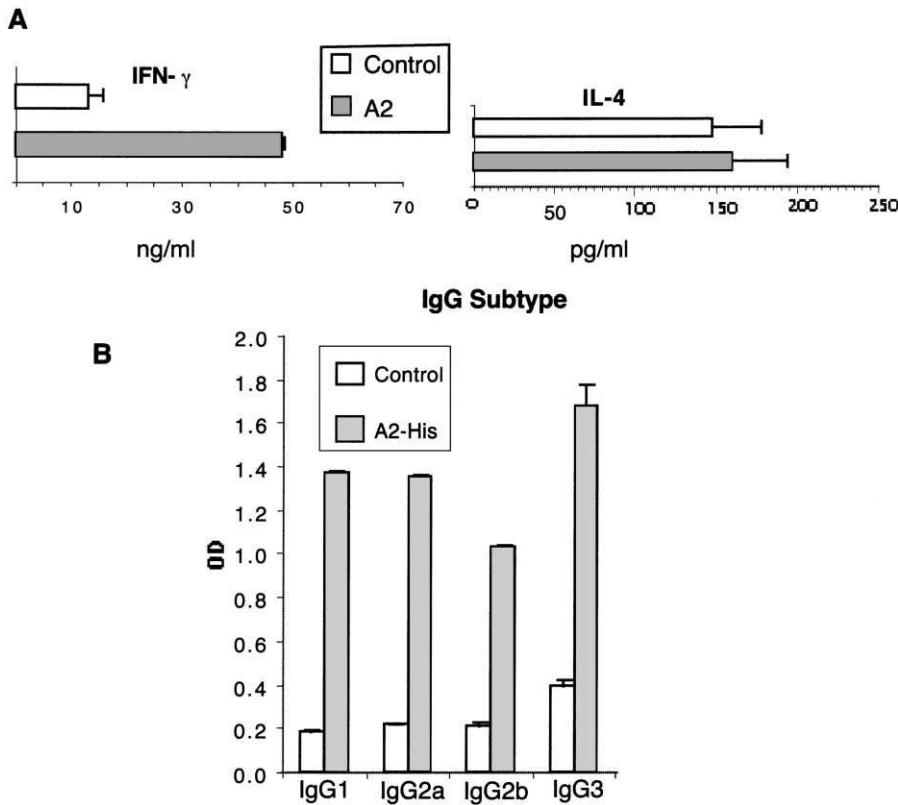


Fig. 4. Panel A, IFN- γ and IL-4 release assay in splenocytes from A2 protein-immunized mice. Mice were immunized with A2. Splenocytes were stimulated with recombinant A2 for 96 h and concentrations of IFN- γ and IL-4 in the culture supernatants was determined. The data is represented as the mean \pm S.E. Each sample was examined in triplicate and these results are representative of two experiments. Note that the IFN- γ and IL-4 are represented on different scales. Panel B, IgG isotype assay. The A2-specific IgG isotype titre was determined by ELISA. The relative subclass titre is represented as OD values and the data is representative of two experiments. Control mice received only adjuvant as described in Section 2.

3.5. Adaptive transfer of splenocytes from A2-vaccinated mice protects against *L. donovani* infection

Protection against *L. donovani* infection is thought to be predominantly T-cell mediated as demonstrated by adaptive transfer of immune spleen cells to naive mice [42]. Thus, adaptive transfer of spleen cells from A2-immunized mice was carried out in both BALB/c and C57BL/6 mice. As shown in the Fig. 5, mice demonstrated a significant level of protection when passively immunized with spleen cells from A2-vaccinated mice in comparison to the control group of mice which received spleen cells from adjuvant-immunized mice. The LDU was reduced by 50% ($P = 0.0215$) and 55% ($P = 0.0044$) for BALB/c and C57BL/6 mice, respectively. These results confirm that irrespective of the strain of mice, A2 antigen passive immunization imparts significant protection against challenge infection.

3.6. Anti-A2 antibodies and complements block amastigote internalization by macrophages *in vitro*

BMMs from BALA/c mice represent an appropriate cell type to measure infection by *Leishmania* *in vitro*. We

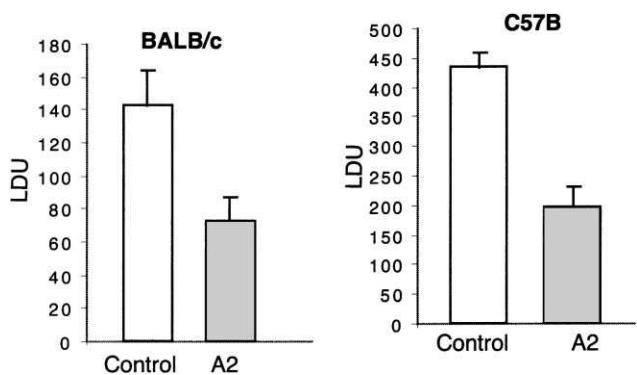


Fig. 5. Infection levels in mice challenged with *L. donovani* following adoptive transfer of splenocytes from A2-vaccinated mice. BALB/c and C57BL/6 mice were immunized with A2 protein and 3 weeks following the final boost, spleen cells were collected and transferred to naive mice. One week after the transfer, mice were challenged with *L. donovani* promastigotes and 4 weeks after the challenge infection, mice were killed and LDU was calculated from liver biopsies. The mean LDU \pm S.E. is shown ($n = 4$ mice per group). This result is the representative of two independent experiments.

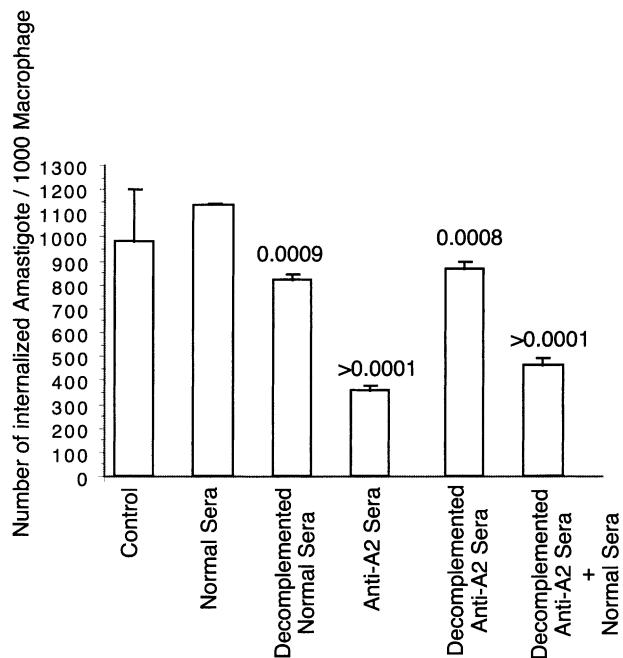


Fig. 6. Internalization of amastigotes in the presence of anti-A2 sera. BMMs (10^6 cells/ml) were infected with amastigotes for 24 h and internalization of parasites were measured. Prior to infection, the amastigotes were incubated with indicated sera samples or control (no-sera). The result is represented as number of internalized amastigotes per 1000 macrophages. The *P*-values of Student's *t*-test indicated on each bar are in comparison with the values obtained from normal sera treatment. The mean \pm S.E. is shown ($n = 3$). This result is the representative of three independent experiments.

therefore used this *in vitro* model system to measure infection with *L. donovani* amastigotes in macrophages in the presence of anti-A2 antibodies. This was carried out both in the presence and absence of viable complement. BMMs were incubated with the same number of *L. donovani* amastigotes in the presence of 1:50 dilution of the various sera combinations. As shown in Fig. 6, there was a significant reduction in *L. donovani* infection in the presence of anti-A2 sera. However, when the anti-A2 sera was decomplemented, the internalization of amastigotes was significantly increased to levels similar to the control. When decomplemented anti-A2 sera was reconstituted with normal mouse sera as a source of complement the internalization was again significantly reduced. Similar observations were made using anti-A2 monoclonal antibodies where the addition of complement to these antibodies also reduced the levels of infection (data not shown). These data argue that the A2 antisera in the presence of complement can reduce the viability of amastigotes resulting in a reduction in infection of macrophages.

4. Discussion

L. donovani complex is the lethal causative agent of VL in humans. Antigens stimulating cellular immune response

to *Leishmania* infection have considerable importance for their potential as vaccines against leishmaniasis. The major observation in this study is that immunization with recombinant A2 protein resulted in significant protection of BALB/c mice against *L. donovani* infection. The protective response generated by recombinant A2 protein immunization was associated with a mixed Th1/Th2 response, production of IFN- γ in response to A2 antigen, and an anti-A2 humoral response. These results argue that protection against *L. donovani* infection by A2 immunization did not appear to be biased towards either Th1 or Th2. Protection of passively-immunized mice against challenge infection with spenocytes from A2-immunized mice indicated that protection is mainly due to the cellular immune response and this was observed in both BALB/c and C57BL/6 mice.

VL disease progression in human and experimental mice is associated with an increased titre of polyclonal and parasite-specific antibodies [43–46], absence of delayed type of hypersensitivity (DTH), and increased IFN- γ production by PBMCs [37]. Various experimental and clinical studies have shown that parasite-specific cellular responses are required to protect against Kala-azar [47]. With respect to CL, it has been documented that susceptible to infection in susceptible BALB/c mice is associated with proliferation of CD4 $^{+}$ Th2 cells, which result the secretion of IL-4, IL-5, IL-6 and IL-10, leading to a humoral response [48–51]. In contrast, CL infection in resistant C57BL/6 mice results in proliferation of the CD4 $^{+}$ Th1 subpopulation of T cells, leading to a cell mediated immune response via release of IFN- γ , TNF- α and IL-2, which results in a recovery from infection [48–51].

Unlike for CL infections, vaccine induced protection against VL in experimental murine model does not correlate with the differential production of Th1 and Th2 cytokines [52] as we observed in this report. Moreover, both Th1 and Th2 responses coexisted in cured VL patients [53,54] and a consistent correlation between IFN- γ production and disease protection in human and experimental VL is observed [38]. These previous findings are consistent with our observations that protection with A2 antigen is due to IFN- γ production where both Th1 and Th2 response coexist.

As described in Section 1, the A2 amastigote-specific protein of *L. donovani* is a virulence factor [32]. Electron microscopy of *L. donovani* amastigotes stained with gold beads conjugated with a anti-A2 monoclonal antibody shows A2 protein throughout the amastigote and also is present on or along the amastigote surface as concentrated patches [J Clos, personal communication]. Results from the *in vitro* macrophage infection with amastigotes in presence of anti-A2 sera (Fig. 6) may indicate a potential role of A2 in the binding and internalization of amastigotes by macrophage. The internalization of promastigotes into macrophages has been shown to mediate by mannose receptor [55], fibronectin receptor [56], complement receptor CR1 and CR3 [55,57]. However, internalization of amastigotes into the macrophages remains controversial. Some

reports showed no contribution of opsonins to the infection of amastigotes [58], whereas other studies have demonstrated role of both Fc receptor and CR3 for macrophage infection [59,60]. However, the in vitro internalization experiment reported within this study may indicate a possible role of A2 in internalization process and thus argue that A2 immunization could protect against inter-macrophage infection and disease progression.

In summary, there are a number of factors which support the argument that A2 proteins could represent an effective vaccine against VL. These proteins contain multiple repeat subunits and are expressed at much higher levels in amastigotes than in promastigotes [30,32]; A2 deficient *L. donovani* resulted in a avirulent phenotype [32]; A2-specific antibodies are present in Kala-azar patients' sera [31]. Taken together, the A2 immunization (both direct and passive) shows protection against visceral infection which is associated with IFN- γ production, coexistence of both Th1 and Th2, strong humoral response and reduced internalization of amastigotes into macrophages. These data argue that A2 is a strong vaccine candidate against VL or Kala-azar.

Acknowledgements

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Generation and evaluation of A2-expressing *Lactococcus lactis* live vaccines against *Leishmania donovani* in BALB/c mice

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Leishmaniasis is a parasitic disease affecting over 12 million individuals worldwide. As current treatments are insufficient, the development of an effective vaccine is a priority. This study generated and assessed the efficacy of *Leishmania* vaccines engineered from the non-colonizing, non-pathogenic Gram-positive bacterium *Lactococcus lactis*. A truncated, codon-optimized version of the A2 antigen from *Leishmania donovani* was engineered for expression in *Lactococcus lactis* in three different subcellular compartments: in the cytoplasm, secreted outside the cell or anchored to the cell wall. These three A2-expressing *Lactococcus lactis* strains were tested for their ability to generate A2-specific immune responses and as live vaccines against visceral *Leishmania donovani* infection in BALB/c mice. Subcutaneous immunization with live *Lactococcus lactis* expressing A2 anchored to the cell wall effectively induced high levels of antigen-specific serum antibodies. It was demonstrated that *Lactococcus lactis*-based vaccines are a feasible approach in the generation of live vaccines against leishmaniasis. The *Lactococcus lactis* strains generated in this study provide an excellent foundation for further studies on live bacterial vaccines against leishmaniasis and other pathogens.

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INTRODUCTION

The parasite *Leishmania* affects over 12 million individuals worldwide and manifests as three forms of disease: cutaneous, mucocutaneous and visceral leishmaniasis, the last usually being fatal (reviewed by Kedzierski *et al.*, 2006). Current treatments are insufficient and consist primarily of highly toxic chemotherapy. Furthermore, there are now many instances of parasite resistance to the available treatments (Kedzierski *et al.*, 2006). Therefore, a vaccine that can prevent the occurrence and reduce the spread of the disease is a priority in this field. The ability to develop protective immunity against leishmaniasis following deliberate inoculation with live parasites, known as leishmanization, has demonstrated that the creation of an effective vaccine against leishmaniasis is possible (Khamesipour *et al.*, 2005).

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); cwa, cell-wall anchoring; FBS, fetal bovine serum; HRP, horseradish peroxidase; IFN- γ ; gamma interferon; IL, interleukin; LDU, Leishman–Donovan units; p.i., post-infection; SLA, soluble *Leishmania* antigen; Th, T helper; wt, wild-type.

A potential vaccine antigen against leishmaniasis is the amastigote-specific A2 family of proteins, which were identified in *Leishmania donovani* (Charest & Matlashewski, 1994). The A2 proteins contain an N-terminal signal peptide, followed by a variable number of repeats of a 10 aa sequence and a 7 aa C-terminal region (Charest & Matlashewski, 1994). As a result, in *Leishmania donovani*, wild-type A2 proteins (wta2) vary in size from 45 to 100 kDa, representing 40–90 repeats (Fig. 1a). Analysis of *Leishmania* genomic DNA has shown that A2 is found in strains that cause both visceral (*Leishmania donovani*, *Leishmania infantum* and *Leishmania chagasi*) and cutaneous (*Leishmania amazonensis* and *Leishmania mexicana*) leishmaniasis (Garin *et al.*, 2005; Ghedin *et al.*, 1997). However, the A2 gene is present in *Leishmania major* as non-expressed pseudo-genes (Zhang *et al.*, 2003). A2 contributes to the ability of some *Leishmania* parasites to infect visceral organs and is therefore considered a putative virulence factor (Garin *et al.*, 2005; Zhang & Matlashewski, 2001; Zhang *et al.*, 2003). This antigen was found to be protective in recombinant protein vaccines in various vaccine mouse models, including against cutaneous and visceral leishmaniasis caused by *Leishmania amazonensis* (Coelho *et al.*, 2003).

and *Leishmania donovani* (Ghosh *et al.*, 2001a, b), respectively. In addition, the A2 antigen was protective in mice when expressed as a recombinant adenovirus vaccine (Resende *et al.*, 2008) and when expressed in the non-pathogenic *Leishmania tarentolae* strain as a live heterologous vaccine (Mizbani *et al.*, 2009), and was shown to provide some protection in dogs as a recombinant protein vaccine (Fernandes *et al.*, 2008).

Lactococcus lactis is an industrially important Gram-positive lactic acid bacterium that is frequently used in the preparation of fermented foods (Beresford *et al.*, 2001). As this bacterium is found in and consumed as part of various food products, we know that it is non-pathogenic and non-colonizing, and therefore it was given Generally Recognized As Safe (GRAS) status by the US Food and Drug Administration (Casalta & Montel, 2008; FDA, 1995). Recently, *Lactococcus lactis* was used as a live bacterial vector to deliver various biological molecules (Steidler, 2003). Studies in which *Lactococcus lactis* was engineered to express vaccine antigens such as fragment C of the tetanus toxin (Robinson *et al.*, 2004), the E7 antigen of human papilloma virus (Cortes-Perez *et al.*, 2003) and many other bacterial, viral and parasitic antigens have shown that this is an effective strategy to generate live heterologous vaccines (Detmer & Glenting, 2006). Furthermore, we have demonstrated that *Lactococcus lactis* exhibits innate inflammatory effects and the ability to modulate dendritic cell maturation, which indicate a capacity for adjuvanticity (Yam *et al.*, 2008). These properties strengthen the rationale for using *Lactococcus lactis* as a live vaccine vector.

In this study, we engineered strains of *Lactococcus lactis* to express a codon-optimized and truncated form of the *Leishmania* A2 protein at different subcellular locations: in the cytoplasm, secreted outside the cell and anchored to the cell wall. These strains of A2-expressing *Lactococcus lactis* were tested as live bacterial vaccines against visceral *Leishmania donovani* infection in BALB/c mice. We demonstrate that *Lactococcus lactis*-based vaccines are a feasible approach in the generation of live vaccines against leishmaniasis.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains DH5 α (Invitrogen) and BL21(DE3) (Novagen EMD Chemicals) were used for DNA cloning and purification of His-tagged proteins, respectively, and were grown with shaking in LB broth (Wisent) at 37 °C. *Lactococcus lactis* subsp. *cremoris* NZ9000 (Kuipers *et al.*, 1998) was grown without shaking in M17 medium (Oxoid) with 0.5% glucose (GM17) at 30 °C. This strain of *Lactococcus lactis* is a plasmid-free derivative of the dairy starter strain NCDO71 that is suitable for use as a live vaccine vector (Mierau & Kleerebezem, 2005). Antibiotics were added at the following concentrations: 100 µg ampicillin ml⁻¹ and 300 µg spectinomycin ml⁻¹.

Plasmid construction. The pDL278 plasmid (LeBlanc *et al.*, 1992) was used for A2 expression in *Lactococcus lactis*. A nisin-controlled gene expression system was selected, as higher levels of heterologous

protein are achieved with an inducible rather than a constitutive promoter (Bermúdez-Humarán *et al.*, 2004). The nisin-inducible promoter, P_{nisA} (Kuipers *et al.*, 1995), was PCR amplified to add an NsiI restriction site at the starting ATG (Table 1) and was cloned into pDL278 at the unique EcoRI site (Table 2, pDL-P_{nisA}). A 25 bp spacer region containing a unique BsgI restriction site was cloned into the NsiI site of the promoter (Table 1, annealed oligonucleotides; Table 2, pDL-P_{nisAB}). The wild-type A2 from *Leishmania donovani* consists of an N-terminal leader sequence followed by tandem repeats of a 10 aa dominant epitope. The 10 aa wild-type A2 repeat was codon optimized for overexpression in *Lactococcus lactis*; of the ten amino acids, eight were coded by the most rarely used codon in *Lactococcus lactis*. All codons were exchanged with the most commonly used counterpart in *Lactococcus lactis*. The first A2 repeat was generated by a pair of complementary oligonucleotides (Table 1) cloned downstream of the P_{nisA} promoter using the BsgI restriction enzyme, which cuts 12/14 nt upstream of its recognition site (Table 2, pDL-P_{nisA}-cytoA2₁). Subsequently, annealed oligonucleotide pairs corresponding to two A2 repeats (Table 1) were consecutively added at the BsgI site to generate our synthetic A2 protein (Table 2, pDL-P_{nisA}-cytoA2₃). The largest construct stably isolated in *E. coli* consisted of ten tandem repeats of the 10 aa A2 epitope (A2₁₀). The A2₁₀ gene (315 bp, 105 aa) located directly downstream of the P_{nisA} promoter corresponded to cytoplasmic expression in *Lactococcus lactis* (Table 2, pDL-P_{nisA}-cytoA2₁₀).

The secretion signal of Usp45 from *Lactococcus lactis* (125 bp) was PCR amplified from pCWA:E7 (Table 1), kindly provided by P. Langella (Le Loir *et al.*, 1998; Ribeiro *et al.*, 2002), and was cloned into the NsiI site between the promoter and the A2 gene to direct protein expression into the culture medium (Table 2, pDL-P_{nisA}-secA2₁₀).

Finally, to direct A2 to be anchored to the cell wall of *Lactococcus lactis*, the cell-wall anchoring (cwa) domain of the M6 protein of *Streptococcus pyogenes* (475 bp) (Piard *et al.*, 1997; Ribeiro *et al.*, 2002) was added to the 3' end of the A2 gene, in addition to the 5'-end secretion signal. A unique NheI restriction site was engineered at the 3' end of the A2 gene. A pair of annealed oligonucleotides corresponding to one A2 repeat followed directly by the NheI site (Table 1) was cloned into the BsgI site of a construct containing only nine tandem repeats of the 10 aa A2 epitope. This resulted in the same A2 gene as above but with a unique NheI site (Table 2, pDL-P_{nisA}-cytoA2_{10N}). The cwa domain was PCR amplified from pCWA:E7 to include flanking NheI sites (Table 1) and was cloned downstream of A2. The addition of the secretion signal and the cwa domain 5' and 3' to the gene, respectively, directed A2 protein expression to be anchored to the cell wall (Table 2, pDL-P_{nisA}-cwaA2₁₀).

To create a His-tagged version of A2 for protein purification, the cytoA2 gene was excised with NsiI and BsgI and blunt cloned into the plasmid pET-16b (Table 2; Novagen) digested with XbaI and BamHI. This strategy maintained the ORF of the His tag with the gene and resulted in the entire synthetic A2 protein, consisting of ten 10 aa repeats, attached to an N-terminal 10 × His tag of the plasmid (Table 2, pET-A2₁₀). The pET-16b-based plasmid containing the His-tagged version of the wild-type A2 gene from *Leishmania donovani* (Table 2, pET-wtA2) has been described previously (Charest & Matlashewski, 1994).

Restriction enzymes were purchased from New England Biolabs. All plasmid constructs were confirmed by DNA sequencing and transferred into *Lactococcus lactis* by electroporation, as described previously (Wells *et al.*, 1993).

Protein expression, purification and immunoblotting. His-tagged protein purification from *E. coli* was performed according to the manufacturer's protocol (Novagene). Saturated overnight cultures of *E. coli* were diluted 1:100 in fresh medium and grown until they reached an OD₆₀₀ of 0.4–0.5. Protein expression was induced by the

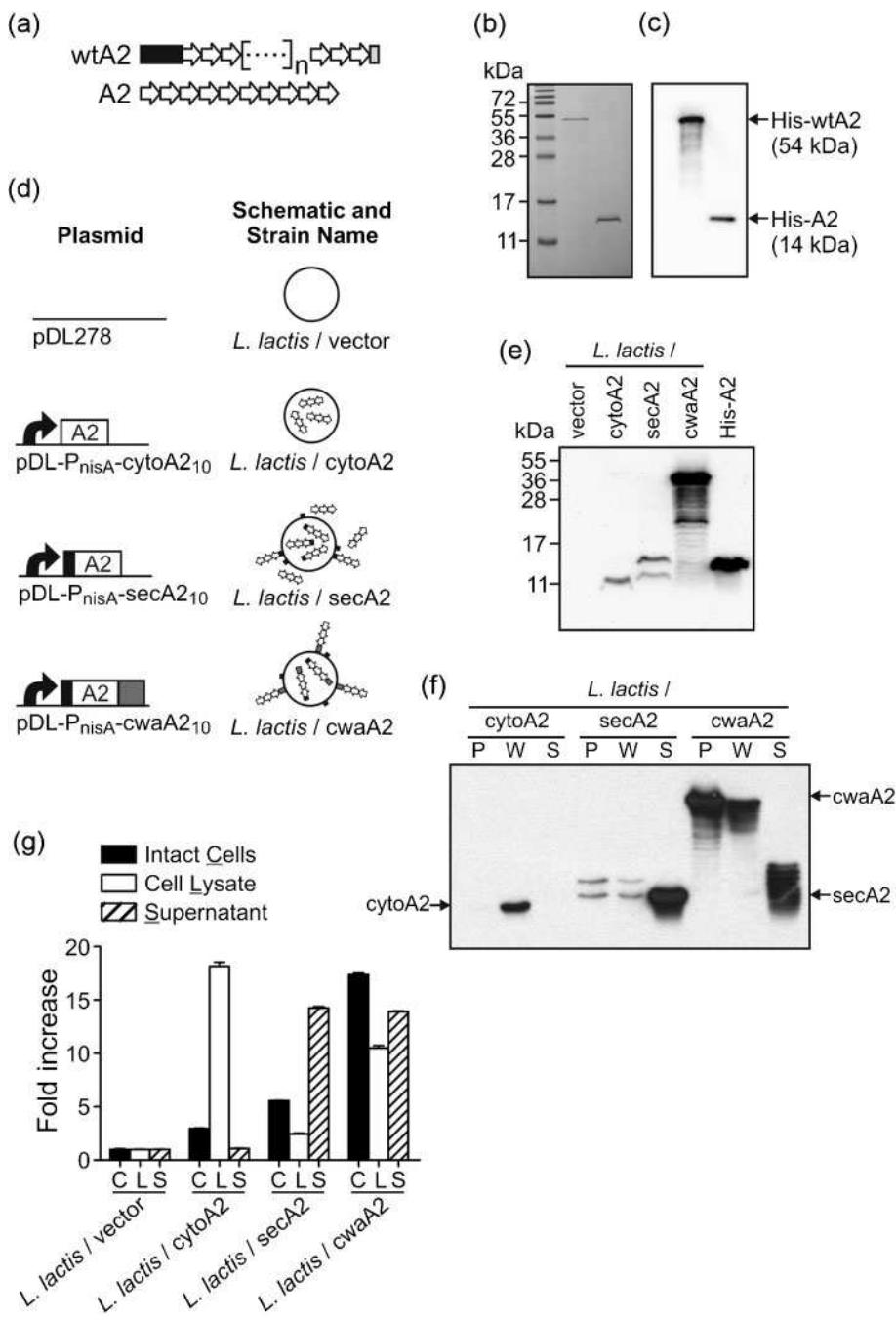


Fig. 1. Generation, expression and localization of a synthetic A2 protein in *E. coli* and in three subcellular compartments of *Lactococcus lactis*, and comparison of the wild-type A2 gene from *Leishmania* spp. (wtA2) and our synthetic A2 gene (A2), which was expressed in *E. coli* and *Lactococcus lactis*. Strains of *Lactococcus lactis* expressing A2 in the cytoplasm, secreted into the supernatant and anchored to the cell wall were generated in this study. (a) Schematic representation of the wtA2 and A2 genes. Filled box, putative secretion signal; open arrows, 10 aa dominant epitope of A2; shaded box, 7 aa C-terminal region. (b) Coomassie blue-stained SDS-polyacrylamide gel of His-purified wtA2 (His-wtA2) and synthetic A2 (His-A2) expressed in *E. coli*. (c) Western blot analysis of the purified A2 proteins using A2-specific antibodies. (d) *Lactococcus lactis* plasmid constructs, schematic representations of expression strategies and strain names. Arrow, nisin-inducible promoter (P_{nisA}); open box, synthetic A2 gene; filled box, Usp45 secretion signal; shaded box, M6 cell-wall anchoring domain. (e) Western blot analysis of total protein extracts of A2-expressing strains of *Lactococcus lactis*. (f) Cell fractionation and Western blot analysis of A2 expression in *Lactococcus lactis*. P, Protoplast; W, cell-wall fraction; S, supernatant; cytoA2, cytoplasmic A2; secA2, secreted A2; cwaA2, cell-wall-anchored A2. (g) Whole-cell ELISA analysis of the localization of A2 in *Lactococcus lactis*. Data presented are the fold increase compared with *Lactococcus lactis* containing the empty vector (*Lactococcus lactis*/vector) for each compartment. C, Intact cells; L, heat-killed cell lysate; S, culture supernatant.

Table 1. Primers used in this study

F, forward; R, reverse; T, top; B, bottom.

| Name | Purpose | Sequence (5'→3')* |
|-----------------------|--|--|
| P _{nisA} | To PCR amplify the nisin promoter | F: CCGGAATTTCAGTCGACCTAGTCTTATAACTA TACTGACAATAG (<i>EcoRI</i>) R: TGCGGAATTCAATGCATTGGAGTGCCCTCCTT ATAATTTAT-5' (<i>EcoRI, NsiI</i>) |
| BsgI spacer | To add stop codons and <i>BsgI</i> restriction site | T: TAATAAGGACCGTCTGCCTGCACTGCA (<i>BsgI</i>) B: GTGCAGGACGGTCCTTATTATGCA-5' (<i>BsgI</i>) |
| First A2 | To add the first A2 repeat | T: TCAATCAGTTGGTCCATTATCAGTTGGTCCA B: GACCAACTGATAATGGACCAACTGATTGATG |
| A2 doublet | To add subsequent doublet of A2 repeats | T: CAATCAGTTGGTCCATTATCAGTTGGTCCA ATCAGTTGGTCCATTATCAGTTGGTCCA B: GACCAACTGATAATGGACCAACTGATTGTGGA CCAACGTATAATGGACCAACTGATTGTG |
| Sec signal | To PCR amplify the Usp45 secretion signal | F: CCAATGCATAAAAAAAAGATTATCTCAGCTAT (<i>NsiI</i>) R: CCAATGCATATCTGAGTTGTGTCAGCG (<i>NsiI</i>) |
| A2 repeat <i>NheI</i> | To add one A2 repeat and <i>NheI</i> restriction site | T: CAATCAGTTGGTCCATTATCAGTTGGTCCA CAATCAGTTGGTCCATTATCAGTTGGTCCA B: GCTAGCAATGGACCAACTGATTGTGACCAAC TGATAATGGACCAACTGATTGTG (<i>NheI</i>) F: CCAATGCATCGCTAGCTTTAGAAGAAGCAAACA (<i>NheI</i>) R: TGAGTGCTAGCTTAGTTCTCTTTGCG (<i>NheI</i>) |
| CWA | To PCR amplify the cell-wall anchoring domain from the M6 protein of <i>Streptococcus pyogenes</i> | |

*Underlined nucleotides signify locations of restriction sites (enzyme indicated in parentheses).

addition of 1 mM IPTG and cultures were grown for an additional 3 h.

Saturated overnight cultures of *Lactococcus lactis* were diluted 1:20 in fresh medium and grown to OD₆₀₀ 0.4–0.5. Protein expression was induced by the addition of nisin (10 ng ml⁻¹, maximal subinhibitory concentration) and cultures were grown for an additional 3 h. We found that growth for 3 h, following nisin induction, allowed the highest level of protein accumulation within bacterial cells (data not shown). Following protein expression, cultures (2 ml) were subjected to a total cell protein extract or a cell fractionation protocol, essentially as described previously (Piard *et al.*, 1997). In both cases, bacterial cells were collected by centrifugation (4300 g, 10 min, 4 °C) and washed with TES [10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 25% sucrose]. For the total cell extract, cells were resuspended in TES-LMR (TES with 1 mg lysozyme ml⁻¹, 50 U mutanolysin ml⁻¹ and 0.1 mg RNaseA ml⁻¹) and incubated at 37 °C for 1 h to digest the cell wall. Cells were lysed in TE buffer with 2% SDS and then incubated in boiling water for 5 min. For the cell fractionation, following centrifugation, the culture supernatant was recovered and was precipitated on ice with 10% TCA for 1 h. The protein pellet was collected by centrifugation in a microcentrifuge (13 000 r.p.m., 15 min, 4 °C) and washed with cold acetone. The pellet was dried briefly and resuspended in 50 mM NaOH. The cell pellet was washed with TES, resuspended in TES-LMR and incubated at 37 °C for 1 h, as described above. The protoplast (pellet) was collected by centrifugation (2000 g, 15 min, 4 °C), whilst the supernatant corresponded to the proteins released from the cell wall (cell-wall fraction). Protoplasts were lysed in TE buffer with 2% SDS and then incubating in boiling water for 5 min. The cell-wall fraction was TCA precipitated as for the culture supernatant fraction and resuspended in 50 mM NaOH.

Protein preparations were resolved by 18% SDS-PAGE and stained with Coomassie blue or transferred onto PVDF membranes

(Immobilon-P; Millipore) for Western blotting. Membranes were blotted with A2-specific antibodies, which were generated against the dominant epitope of the wta2 protein and secreted from the C9 hybridoma cell line (Zhang *et al.*, 1996), diluted 1:20 000 in PBS + 1% Tween 20 (Fisher) containing 5% non-fat dried milk. Anti-mouse secondary antibodies against total IgG, conjugated to horseradish peroxidase (HRP; Sigma), were used at a dilution of 1:20 000 in PBS + 1% Tween 20 with 5% non-fat dried milk. Membranes were detected using Immobilon Western Chemiluminescence HRP Substrate (Millipore) and visualized on radiographic film or using the VersaDoc Molecular Imager (Bio-Rad) with QuantityOne software (Bio-Rad).

Preparation of bacteria for whole-cell ELISA or immunizations.

Following nisin induction as described above, *Lactococcus lactis* cultures were washed twice in PBS, and resuspended in PBS + 25% glycerol at 1/25th of the starting volume. Bacterial cultures were aliquotted and stored at -80 °C. The bacterial concentration of one aliquot was determined, and the dilution factor was calculated for the remaining aliquots to obtain the necessary concentration. First, an aliquot of frozen bacteria was allowed to thaw on ice and centrifuged (3000 g, 10 min, 4 °C), the supernatant was removed and the bacterial cells were resuspended in the same volume of PBS. Serial dilutions and c.f.u. counts were performed to determine the bacterial concentration and to calculate the dilution factor to obtain ~10¹⁰ c.f.u. ml⁻¹. This dilution factor was confirmed by thawing another aliquot, which was centrifuged, resuspended in PBS and quantified by serial dilutions and c.f.u. counts. Finally, when live A2-expressing *Lactococcus lactis* was needed for whole-cell ELISA or mouse immunizations, a frozen aliquot was thawed on ice, centrifuged as above and resuspended in PBS to obtain the necessary bacterial concentration, which was again confirmed by serial dilutions and c.f.u. counts. For each batch of *Lactococcus lactis* prepared, correct A2 expression was also confirmed by immunoblotting and whole-cell ELISA.

Table 2. Bacterial strains and plasmids used in this study

| Name | Description | Reference/supplier |
|---|--|-------------------------------|
| Bacterial strains | | |
| <i>E. coli</i> DH5 α | Standard laboratory strain used for DNA cloning and amplification | Invitrogen |
| <i>E. coli</i> BL21(DE3) | Contains the T7 promoter under an IPTG-inducible promoter | Novagen |
| <i>Lactococcus lactis</i> NZ9000 | A plasmid-free derivative of the dairy starter strain NCDO71 that is suitable for use as a live vaccine vector | Kuipers <i>et al.</i> (1998) |
| Plasmids | | |
| pDL278 | <i>E. coli/Lactococcus lactis</i> shuttle plasmid | LeBlanc <i>et al.</i> (1992) |
| pDL-P _{nisA} | The nisin-inducible promoter (P _{nisA}) added to pDL278 | This work |
| pDL-P _{nisA} B | A unique BsgI restriction site added downstream of the promoter in pDL-P _{nisA} | This work |
| pDL-P _{nisA} -cytoA ₂₁ | One 10 aa repeat of the A2 protein added to pDL-P _{nisA} B downstream of the promoter | This work |
| pDL-P _{nisA} -cytoA ₂₃ | Two 10 aa repeats of the A2 protein added to pDL-P _{nisA} -cytoA ₂₁ | This work |
| pDL-P _{nisA} -cytoA ₂₁₀ | Consecutive additions of doublets of the A2 repeat added to pDL-P _{nisA} -cytoA ₂₃ ; the largest stable construct produced contained ten copies of the A2 repeat downstream of the P _{nisA} promoter. | This work |
| pDL-P _{nisA} -secA ₂₁₀ | The Usp45 secretion signal added between the promoter and A ₂₁₀ gene in pDL-P _{nisA} -cytoA ₂₁₀ | This work |
| pDL-P _{nisA} -cytoA ₂₁₀ N | A unique NheI restriction site added to the 3' end of the A ₂₁₀ gene | |
| pDL-P _{nisA} -cwaA ₂₁₀ | The Usp45 secretion signal added at the 5' end and the cell-wall anchoring (cwa) domain of the M6 protein of <i>Streptococcus pyogenes</i> added to the 3' end of the A ₂₁₀ gene in pDL-P _{nisA} -cytoA ₂₁₀ N | This work |
| pET-16b | Plasmid to create N-terminally His-tagged proteins in <i>E. coli</i> | Novagen |
| pET-A ₂₁₀ | The A ₂₁₀ gene from pDL-P _{nisA} -cytoA ₂₁₀ added to pET-16b to create a His-tagged version of A ₂₁₀ | This work |
| pET-wtA2 | His-tagged version of the wild-type A2 from <i>Leishmania donovani</i> | Charest & Matlashewski (1994) |

Whole-cell ELISA. A whole-cell ELISA protocol was developed from previously described methods (Avall-Jääskeläinen *et al.*, 2002; Tsang *et al.*, 1995). Live A2-expressing *Lactococcus lactis* was prepared as above; however, the medium supernatant was retained after nisin induction and stored at -20 °C. Live bacteria were diluted in PBS to 10⁹ c.f.u. ml⁻¹ and a portion was used to prepare heat-killed *Lactococcus lactis* by incubating in boiling water for 1 h. ELISA plates were coated with live and heat-killed bacteria (10⁸ bacteria in 100 µl per well) and supernatant for each *Lactococcus lactis* strain in triplicate at 4 °C overnight. Before and after every step, wells were washed with PBS + 0.5% Tween 20. Wells were blocked with PBS + 1% BSA (blocking solution) for 1 h, and A2-specific antibodies (C9 cell line supernatant, diluted 1:2000 in blocking solution) was allowed to bind for 2 h. Finally, wells were incubated with HRP-conjugated anti-mouse IgG secondary antibody (diluted 1:2000 in blocking solution; Sigma) for 1 h. The presence of A2 protein was detected by the addition of the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma) and plates were read at 405 nm. Data are presented as the fold increase compared with *Lactococcus lactis* containing the empty vector (*Lactococcus lactis*/vector) for each cellular localization of the protein.

Intracellular growth assay. Experiments were performed using a protocol modified slightly from previously described methods (Bahey-El-Din *et al.*, 2008). In brief, 0.5 × 10⁶ J774 macrophage cells were plated in 1 ml RPMI 1640 (Gibco) containing 10% fetal bovine serum (FBS; Wisent) in six-well tissue culture plates (Becton Dickinson) and incubated at 37 °C with 5% CO₂ overnight. Cells were washed with 5 ml medium and co-incubated with 10⁸ *Lactococcus lactis* for 1 h. Cells were then washed twice and incubated in RPMI 1640 containing 20 µg gentamicin (Gibco) ml⁻¹ for an additional 1 h. After a final wash, cells were lysed in 2 ml cold, sterile PBS + 0.1% Triton X-100 (Fisher) and

serial dilutions were plated on GM17 agar plates with or without antibiotic (spectinomycin). Alternatively, a second set of cells was incubated for an additional 6 h after replacement of the gentamicin-containing medium with fresh medium.

Immunizations. Six-week-old female BALB/c mice were purchased from Charles River Laboratories. Groups of mice were immunized subcutaneously on days 0, 14 and 28 with ~2 × 10⁹ c.f.u. live *Lactococcus lactis* expressing A2 in each of the three subcellular localizations (*Lactococcus lactis*/cytoA2, *Lactococcus lactis*/secA2 or *Lactococcus lactis*/cwaA2), *Lactococcus lactis* containing the empty plasmid vector (*Lactococcus lactis*/vector) or PBS only. All immunizations were performed with a total volume of 200 µl. Blood was collected from the lateral saphenous vein on days 7, 21, 35, 56 and 70 after the first immunization. Serum samples were stored at -20 °C until analysis. Mice were challenged with *Leishmania donovani* infection on day 42.

All procedures were in accordance with the guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

Serum antibody analyses. Reciprocal end-point titres of A2-specific IgG, IgG₁ and IgG_{2a} antibodies were determined in serum samples from individual mice. Briefly, ELISA plates were coated with His-A2 purified protein (2.5 µg ml⁻¹; 50 µl per well) at 4 °C overnight. Before and after every subsequent step, wells were washed with PBS + 0.5% Tween 20. Twofold serial dilutions of serum samples were prepared, and added to wells at a starting dilution of 1:50 in PBS + 2.5% FBS. HRP-conjugated anti-mouse antibodies against total IgG, or goat anti-mouse IgG₁ or IgG_{2a} followed by HRP-conjugated anti-goat secondary antibodies (Sigma), were used.

Finally, plates were detected using ABTS substrate and read at 405 nm. The end-point was determined as the highest serum dilution to reach the same absorbance reading as naïve pre-immune sera (1:50 dilutions) plus 2 SD.

Western blotting analysis was performed to confirm the specificity of the ELISA results. Pooled serum samples from each mouse immunization group were immunoblotted (1:100 dilutions in PBS + 1% BSA) against 0.5 µg His-A2 purified protein, which was resolved by 18% SDS-PAGE and transferred to PVDF membrane. Total IgG, IgG₁ or IgG_{2a} A2-specific antibodies in serum were detected using the secondary antibodies as above. Detection was carried out as described for immunoblotting.

Parasite challenge and soluble *Leishmania* antigen (SLA) preparation. *Leishmania donovani* strain 1S2D expressing episomal luciferase (Roy *et al.*, 2000) was maintained at 25 °C in SDM medium supplemented with 10% FBS, as described previously (Abu-Dayyeh *et al.*, 2008). Mice were challenged with *Leishmania* infection 2 weeks after the last immunization (day 42).

Mice were challenged with 1×10^7 (low dose) or 4×10^7 (high dose) late-stationary-phase *Leishmania donovani* promastigotes in 100 µl PBS injected in the tail vein. At 4 weeks post-infection (p.i.), mice were sacrificed and Leishman–Donovan units (LDU) were calculated as described previously (Roy *et al.*, 2000). Briefly, microscope slides of liver impression smears were prepared and stained with Diff–Quick solution (Dade Behring). The LDU was calculated as the number of amastigotes counted per 1000 nucleated cells multiplied by the liver weight (mg). The results shown represent five to ten mice per group for the low-dose challenge and ten mice per group for the high-dose challenge.

To prepare SLA, late-stationary-phase *Leishmania donovani* promastigotes were first resuspended in sterile PBS to a concentration of $\sim 10^8$ parasites ml⁻¹. Parasites were lysed by five cycles of freeze–thawing, then centrifuged in a microcentrifuge (14 000 r.p.m., 4 °C, 15 min). The supernatant (SLA) was collected and protein concentration was determined by a Bradford assay (Bio-Rad).

Ex vivo stimulation of splenocytes. Spleens were excised from mice immunized and challenged with *Leishmania donovani* at 4 weeks p.i. Single-cell suspensions of splenocytes were prepared and adjusted to 2×10^6 cells ml⁻¹ in RPMI 1640 supplemented with 10% FBS, 1 mM penicillin/streptomycin and 0.5 mM β-mercaptoethanol. Splenocytes were plated in 24-well plates at 2×10^6 cells in a total of 2 ml. Cells were stimulated with SLA prepared from *Leishmania donovani* at 50 µg ml⁻¹ for 3 days. Cell culture supernatants were collected and stored at –80 °C until quantification of cytokines by ELISA. The concentrations of gamma interferon (IFN-γ), interleukin 2 (IL-2) and IL-10 were determined using ELISA Ready-SET-Go! Cytokine kits according to manufacturer's protocols (eBiosciences). The results shown represent five mice per group.

Statistical analysis. Statistical significance between groups was determined using the analysis of variance function of the StatView program, version 5.0 (SAS Institute).

RESULTS

Generation of a truncated A2 protein for expression in bacteria

To enhance expression of a *Leishmania* protein in *Lactococcus lactis*, we engineered a codon-optimized version of the gene encoding the A2 antigen. Heterologous gene expression in *Lactococcus lactis* is enhanced by the

replacement of rare bacterial codons (Fuglsang, 2003). Proteins of the A2 family from *Leishmania donovani* consist of an N-terminal leader sequence followed by a variable number of tandem repeats of a 10 aa dominant epitope followed by a 7 aa C-terminal region (Charest & Matlashewski, 1994; Zhang *et al.*, 2003). In comparison, the A2 protein generated for expression in bacteria contained ten copies of the 10 aa repeat (Fig. 1a). The His-tagged purified bacterial A2 protein separated by 18% SDS-PAGE had a size of 14 kDa, which corresponded to the molecular size of this protein, whilst A2 expressed from *Leishmania donovani* cDNA in *E. coli* was 54 kDa (Fig. 1b) (Charest & Matlashewski, 1994). The codon-optimized A2 protein was also recognized by A2-specific antibodies (Zhang *et al.*, 1996) when analysed by Western blotting, similar to the wild-type A2 from *Leishmania donovani* expressed in *E. coli* (Fig. 1c).

Expression and localization of A2 in three different subcellular compartments of *Lactococcus lactis*

A2 expression in *Lactococcus lactis* was directed to different subcellular localizations to determine which presentation strategy stimulated the highest level of protection against leishmaniasis. As described in Methods, A2 was expressed in the cytoplasm (*Lactococcus lactis*/cytoA2), secreted from the bacterial cell (*Lactococcus lactis*/secA2) and anchored to the cell wall (*Lactococcus lactis*/cwaA2) (Fig. 1d).

To confirm the expression levels and localization of A2 in *Lactococcus lactis* following nisin induction, Western blot analyses and a whole-cell ELISA were performed on the three strains of *Lactococcus lactis*. As shown in Fig. 1(e), following nisin induction, A2 protein was detectable in all strains of A2-expressing *Lactococcus lactis* but was absent in the negative-control strain (*Lactococcus lactis*/vector). Furthermore, by cell fractionation and Western blot analysis, the three strains of A2-expressing *Lactococcus lactis* exhibited bands corresponding to A2 in the anticipated fractions (Fig. 1f). A2 protein directed to the cytoplasm (cytoA2, 105 aa, 12 kDa) was detected primarily in the cell-wall fraction (Fig. 1f), but at longer exposures of the Western blot it was also detected in the protoplast fraction. Previous studies have shown that, with this cell fractionation protocol, the ionic charge of proteins and the salt concentration of the growth medium can lead to cytoplasmic proteins appearing in the cell-wall fraction (Dieye *et al.*, 2001). The localization of cytoA2 was confirmed by whole-cell ELISA (Fig. 1g). In comparison with *Lactococcus lactis*/vector, cytoA2 was detected mainly in the cell lysate, minimally on the surface of intact cells and not in the culture supernatant. Therefore, *Lactococcus lactis*/cytoA2 correctly expressed cytoA2 in the cytoplasm. Similar analyses confirmed the expression and correct localization of secA2 and cwaA2 (Fig. 1f, g). The secA2 protein was detectable in all cell fractions; this was expected, as the protein would first be expressed in the cytoplasm with the secretion signal (146 aa, 15 kDa),

which would then be cleaved during processing to direct the protein into the culture supernatant (12 kDa) (Fig. 1f). Whole-cell ELISA results confirmed that secA2 was detectable on the cell surface before processing of the secretion signal but was found mainly in the culture supernatant (Fig. 1g). Finally, with the addition of the cwa domain, the larger size of cwaA2 was seen as expected in the protoplast and cell-wall fractions on the Western blot (304 aa, 34 kDa) (Fig. 1f). Smaller bands of cwaA2 were also detected in the culture supernatant, which may correspond to proteins that were located initially on the cell surface but were sloughed off during renewal of the cell wall. Corresponding with the Western blot data, high levels of cwaA2 were detected on the cell surface of this strain in comparison with *Lactococcus lactis*/vector, as well as in the cell lysate and the culture supernatant (Fig. 1g).

Intracellular fate of *Lactococcus lactis* in the J774 macrophage cell line

To assess infectivity, bacterial fate and plasmid stability in J774 cells, *Lactococcus lactis* was incubated with macrophages for 2 or 8 h. Cells were then lysed and bacterial c.f.u. counts were determined. At both time points, there was no detectable difference in the c.f.u. counts between plasmid-free *Lactococcus lactis* NZ9000 and *Lactococcus lactis* harbouring the different constructs (empty vector, cytoA2, secA2 and cwaA2) (data not shown). This indicated that the higher metabolic burden of protein expression did not affect the infectivity of *Lactococcus lactis* *in vitro*. However, the number of live *Lactococcus lactis* recovered from macrophages decreased two- to fourfold following the additional 6 h period, which suggests that *Lactococcus lactis* bacteria are incapable of long-term survival within macrophages (data not shown; Bahey-El-Din *et al.*, 2008). Finally, we also compared c.f.u. counts of the different strains plated with or without antibiotic to assess plasmid stability. At both time points, we were unable to detect any differences in c.f.u. counts between bacteria grown on plates with or without antibiotic (data not shown), indicating that all plasmids were well maintained within the *Lactococcus lactis* cells.

Antigen-specific humoral responses following administration of A2-expressing *Lactococcus lactis* live vaccines

Mice were immunized by subcutaneous injections of live bacteria at 2-week intervals for a total of three immunizations. Alternatively, mice were immunized with PBS only or *Lactococcus lactis* containing the empty plasmid vector without antigen expression (*Lactococcus lactis*/vector) as negative controls. To determine whether the antigen in the context of *Lactococcus lactis* live vaccines was recognized by the immune system, the strains of A2-expressing *Lactococcus lactis* were evaluated for their ability to generate antigen-specific antibodies. Serum samples were collected from individual mice on alternate weeks from the weeks in which mice were immunized.

Interestingly, only expressed A2 anchored to the cell wall of *Lactococcus lactis* (not cytoplasmic or secreted expression) was able to induce high levels of A2-specific IgG antibodies in serum (Fig. 2a). In mice that were immunized with *Lactococcus lactis*/cwaA2, the end-point A2 titre was as high as 10^4 (Fig. 2a). The specificity of ELISA results was also confirmed by Western blot; only mice immunized with *Lactococcus lactis*/cwaA2 exhibited noticeable production of A2-specific antibodies (Fig. 2b).

The isotype of the antigen-specific antibodies produced is an indicator of the type of immune response generated. The induction of a T-helper 1 (Th1)-type immune response results in an isotype switch to IgG_{2a}, whilst a Th2-type immune response promotes production of IgG₁ (Stavnezer, 1996). Immunization with *Lactococcus lactis*/cwaA2 induced A2-specific antibodies of both isotypes, IgG₁ and IgG_{2a}, which suggested that both Th1 and Th2 immune responses were induced (Fig. 2c). However, the ratio of IgG₁:IgG_{2a} antibodies generated following immunization with *Lactococcus lactis*/cwaA2 was 2.42, suggesting that there was predominantly a Th2-type response against the A2 antigen in the immunized mice when A2 was delivered by *Lactococcus lactis*.

Evaluation of A2-expressing *Lactococcus lactis* live vaccines against *Leishmania donovani* challenge

Next, we assessed the efficacy of A2-expressing *Lactococcus lactis* live vaccines against *Leishmania donovani* infection. The A2 antigen was originally isolated from this strain of *Leishmania*, and immunization with purified A2 protein has been shown previously to protect mice against *Leishmania donovani* infection (Charest & Matlashewski, 1994; Ghosh *et al.*, 2001b). Mice were challenged with a low or high dose of *Leishmania donovani* promastigotes injected intravenously 2 weeks after the final immunization (day 42), and disease was allowed to progress for 4 weeks.

Following challenge with *Leishmania donovani* infection, mice in the negative-control groups (immunized with PBS and *Lactococcus lactis*/vector) demonstrated comparable levels of parasitaemia, as determined by the LDU (Fig. 3). LDU values of both groups of control mice that received the low dose of parasites reached levels above 8000 LDU (Fig. 3a), whilst for the high-dose challenge, the control mice reached ~80 000 LDU (Fig. 3b). Following low-dose challenge, mice that were immunized with *Lactococcus lactis*/cytoA2 and *Lactococcus lactis*/cwaA2 demonstrated a reduction in LDU (Fig. 3a). Interestingly, as seen with the high-dose parasite challenge, mice that were immunized with *Lactococcus lactis*/cytoA2 also exhibited the lowest LDU in comparison with control mice (Fig. 3a, b). Immunization with *Lactococcus lactis*/cytoA2 resulted in reductions of approximately 50 and 40 % in LDU in comparison with control mice after low- and high-dose challenge with *Leishmania donovani*, respectively. However, this reduction in LDU by immunization

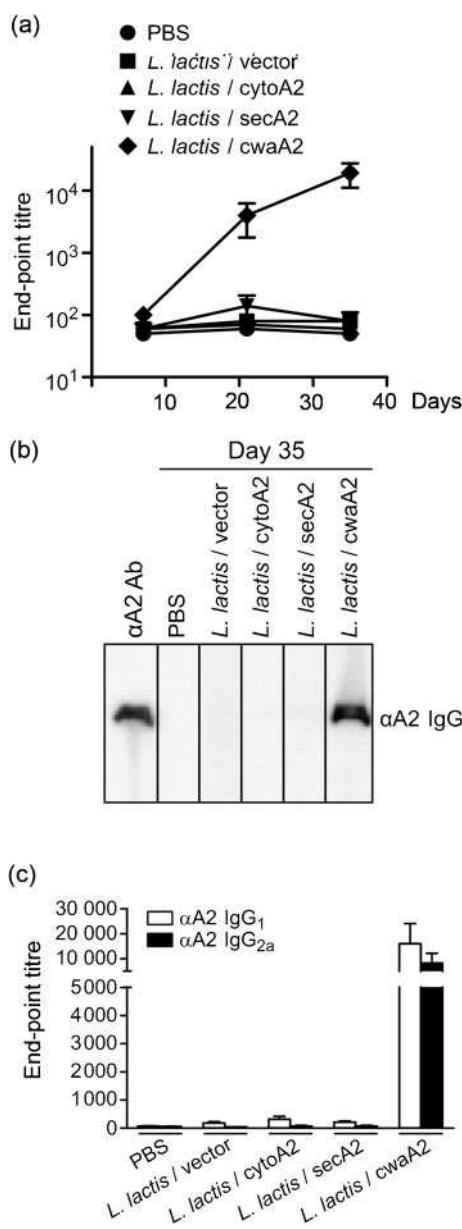


Fig. 2. Antigen-specific humoral responses following administration of A2-expressing *Lactococcus lactis* live vaccines. Detection of anti-A2 total IgG, IgG₁ and IgG_{2a} antibodies in the serum of mice immunized subcutaneously with A2-expressing *Lactococcus lactis* strains. (a) End-point anti-A2 total IgG antibody titres measured by ELISA of serum samples from individual mice taken on days 7, 21 and 35. Mice were immunized on days 0, 14 and 28. (b) Western blot analysis of pooled serum samples taken on day 35 for each immunization group. Each pooled sample was immunoblotted against 0.5 µg His-A2 on strips of PVDF membrane. αA2 Ab, control anti-A2 antibody. (c) End-point anti-A2 total IgG₁ and IgG_{2a} antibody titres measured by ELISA of serum samples from individual mice taken on day 35. Data are presented as means ± SEM of five mice per group and are representative of two independent experiments.

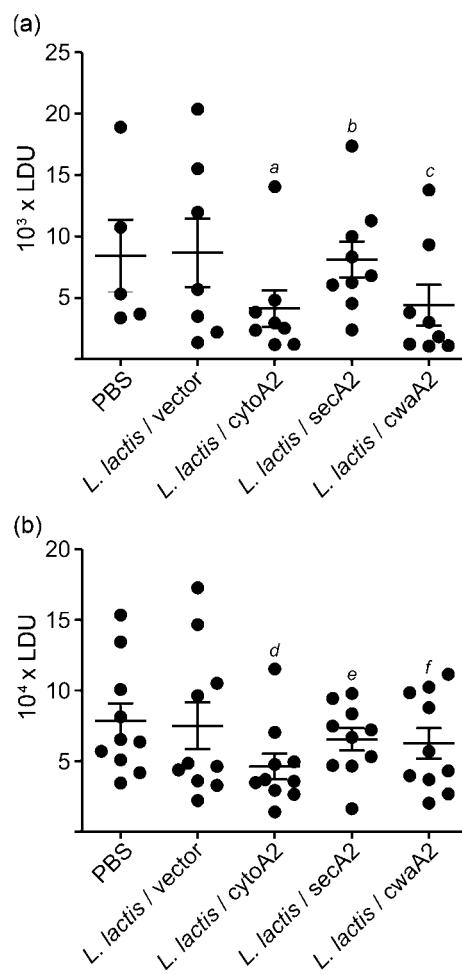


Fig. 3. Protection from A2-expressing *Lactococcus lactis* live vaccines against *Leishmania donovani* challenge. Mice immunized subcutaneously with A2-expressing *Lactococcus lactis* live vaccines were challenged with 1×10^7 (low dose) (a) or 4×10^7 (high dose) (b) of *Leishmania donovani* parasites injected intravenously. Liver parasitaemia was determined at 4 weeks p.i. by calculating LDU values. Data are presented as means ± SEM of five to ten mice per group for the low-dose challenge and ten mice per group for the high-dose challenge. Each dot represents the results for one mouse. P values are given compared with PBS and *Lactococcus lactis*/vector, respectively: a, $P=0.1720$ and $P=0.1128$; b, $P=0.9221$ and $P=0.8405$; c, $P=0.2008$ and $P=0.1358$; d, $P=0.0590$ and $P=0.0880$; e, $P=0.4377$ and $P=0.5594$; f, $P=0.3435$ and $P=0.4499$.

with *Lactococcus lactis*/cytoA2 did not reach statistical significance.

Antigen-specific immune responses of A2-expressing *Lactococcus lactis* live vaccines after *Leishmania donovani* challenge

Blood samples from individual mice were collected at 2 and 4 weeks after mice were challenged with *Leishmania*

donovani infection (at days 56 and 70 following the initial immunization). Similar to the results before challenge, only mice that were immunized with *Lactococcus lactis*/cwaA2 induced high levels of A2-specific antibodies (Fig. 4a), which reached titres above 10^5 . Again, the specificity of the ELISA results was confirmed by Western blotting and demonstrated that A2-specific antibodies were indeed only produced in mice immunized with *Lactococcus lactis*/cwaA2 (Fig. 4b). Furthermore, mice that received the *Lactococcus lactis*/cwaA2 live vaccine produced antigen-specific antibodies of both IgG₁ and IgG_{2a} isotypes, indicating that there was an induction of both Th1- and Th2-type immune responses (Fig. 4c). However, the ratio of A2-specific IgG₁:IgG_{2a} antibodies was 4.67, which was higher than that observed for the serum samples taken before mice were challenged with *Leishmania* infection, suggesting that a stronger Th2-type immune response was induced.

To clarify further the type of immune response produced, at the end of the low-dose *Leishmania donovani* challenge (4 weeks p.i.), splenocytes from individual mice were isolated and then restimulated with SLA. Splenocytes were restimulated with SLA to investigate the immune response against the entire parasite. Cytokines from tissue culture supernatants were quantified by ELISA. Interestingly, following restimulation with SLA, splenocytes from all animals produced IFN- γ at levels higher than unstimulated samples (Fig. 5a). In addition, mice that were immunized with *Lactococcus lactis* expressing cytoA2 and cwaA2 live vaccines expressed even higher levels of IFN- γ . Following the same trend as seen with IFN- γ , splenocytes from mice that received *Lactococcus lactis*/cytoA2 and *Lactococcus lactis*/cwaA2 induced significant levels of IL-10 following SLA restimulation in comparison with PBS (Fig. 5b). However, in comparison with *Lactococcus lactis*/vector, the levels of IL-10 induced by *Lactococcus lactis*/cytoA2 and *Lactococcus lactis*/cwaA2 were not significant. In contrast, splenocytes from mice that received *Lactococcus lactis* live vaccines all induced low levels of IL-4 following restimulation with SLA; however, these were near or below the limit of detection of the assay (data not shown). Finally, both unstimulated and SLA-stimulated splenocytes from all mice produced detectable levels of IL-2 (Fig. 5c). Again, the same trend was observed, in that splenocytes from mice that received *Lactococcus lactis*/cytoA2 and *Lactococcus lactis*/cwaA2 appeared to induce slightly higher levels of IL-2.

DISCUSSION

The objective of this study was to compare different methods of antigen expression and delivery by live *Lactococcus lactis*, and to test *Lactococcus lactis*-based vaccines for protection against *Leishmania donovani* infection in BALB/c mice. We generated a truncated A2 protein encoded by a synthetic gene that was optimized for expression in bacteria. For our synthetic A2 gene, all codons were exchanged for their most common counterparts in

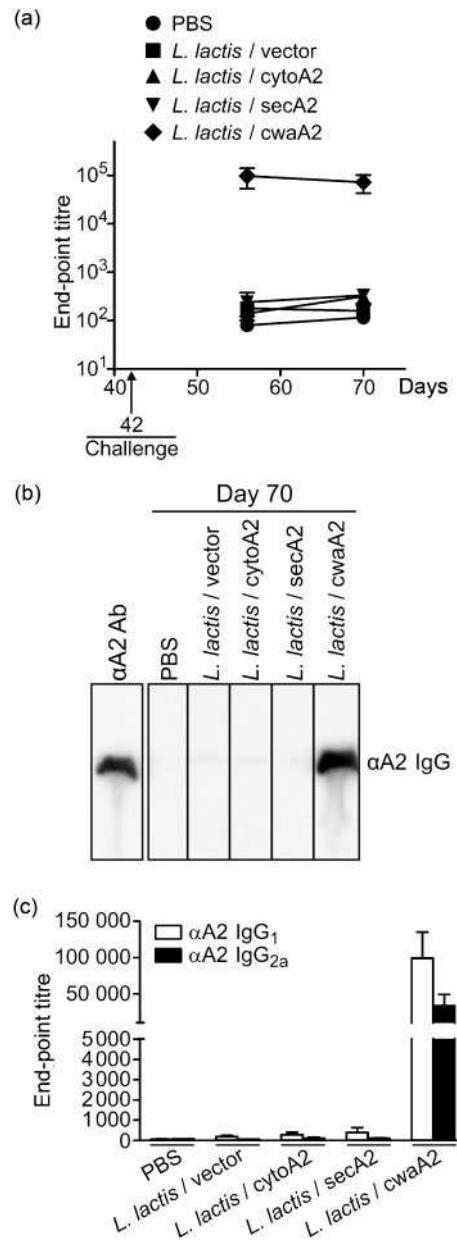


Fig. 4. Antigen-specific humoral responses following challenge with *Leishmania* infection, and detection of anti-A2 total IgG, IgG₁, and IgG_{2a} antibodies in serum of mice following immunization with A2-expressing *Lactococcus lactis* live vaccines and challenge with *Leishmania donovani* infection. (a) End-point anti-A2 total IgG antibody titres measured by ELISA of serum samples from individual mice taken on days 56 and 70 after the initial immunization. (b) Western blot analysis of pooled serum samples taken on day 70 for each immunization group. Each pooled sample was immunoblotted against 0.5 µg His-A2 on strips of PVDF membrane. αA2 Ab, control anti-A2 antibody. (c) End-point anti-A2 total IgG₁ and IgG_{2a} antibody titres measured by ELISA of serum samples from individual mice taken on day 70. Data are presented as the means ± SEM of six mice per group and are representative of two independent experiments.

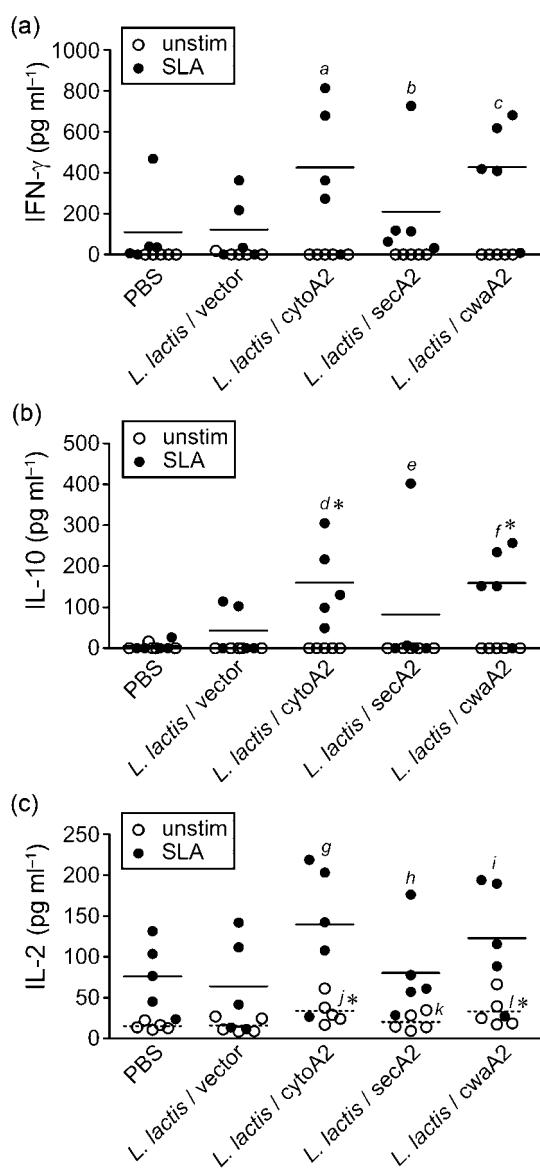


Fig. 5. Splenocyte cytokine responses following challenge with *Leishmania donovani* infection. Splenocytes of mice immunized subcutaneously with A2-expressing *Lactococcus lactis* live vaccines and challenged with low-dose *Leishmania donovani* parasites were restimulated *ex vivo* with SLA. The concentration of cytokines IFN- γ (a), IL-10 (b) and IL-2 (c) in splenocyte culture supernatants was determined after 3 days of stimulation. Data are presented as means \pm SEM of five mice per group. *P* values for the SLA-stimulated samples are given compared with PBS and *Lactococcus lactis*/vector, respectively: *a*, *P*=0.0646 and *P*=0.0752; *b*, *P*=0.5390 and *P*=0.5913; *c*, *P*=0.0636 and *P*=0.0741; *d*, *P*=0.0316 and *P*=0.0966; *e*, *P*=0.2652 and *P*=0.5690; *f*, *P*=0.0327 and *P*=0.0996; *g*, *P*=0.1235 and *P*=0.0705; *h*, *P*=0.9193 and *P*=0.6893; *i*, *P*=0.2507 and *P*=0.1529. *P* values for the unstimulated (IL-2) samples are given compared with PBS and *Lactococcus lactis*/vector, respectively: *j*, *P*=0.0429 and *P*=0.0525; *k*, *P*=0.5667 and *P*=0.6377; and *l*, *P*=0.0471 and *P*=0.0576. *, *P*<0.05 (see above for details).

Lactococcus lactis. Originally, eight of the ten amino acids of the A2 repeat were coded by the most rarely used codon in *Lactococcus lactis*. Bioinformatic analysis of *Lactococcus lactis* gene expression has shown that highly expressed genes have a different codon bias in comparison with seldom-expressed genes (Fuglsang, 2003); therefore, codon optimization was predicted to enhance expression of our synthetic A2 gene. We engineered constructs that expressed A2 in the cytoplasm, secreted it outside the cell and anchored it to the cell wall of *Lactococcus lactis*. These general molecular approaches will be useful for generating future *Lactococcus lactis* live vaccines using different *Leishmania* antigens alone or in combination with A2.

According to our Western blot and whole-cell ELISA analyses, all the A2-expressing strains of *Lactococcus lactis* expressed the antigen correctly in the expected compartment. However, we also detected cwaA2 in the supernatant of this strain. This is unlike previous studies, where expressed antigens that were anchored to the cell wall of *Lactococcus lactis* did not appear in the supernatant according to Western blot results (Bermúdez-Humáran *et al.*, 2004; Medina *et al.*, 2008; Scavone *et al.*, 2007). In contrast, when the L7/L12 antigen of *Brucella abortus* was similarly directed to expression on the cell wall of *Lactococcus lactis*, it was also detected by Western blot in the supernatant (Ribeiro *et al.*, 2002). We hypothesize that this is a result of the stability of the antigen in the growth medium, as there was no correlation with antigen size leading to surface degradation. This difference may also be a result of the conditions used for protein induction. Previous studies using the nisin-inducible promoter induced antigen expression for 1–2 h. However, for our studies, we induced the expression of A2 for 3 h. We found that a longer induction time was able to produce more protein and led to more accumulated antigen within each bacterial cell before its use as a live vaccine. However, as *Lactococcus lactis* produces lactic acid as it grows, a longer growth period would lead to a more acidic medium, which may also cause increased protein degradation on the cell surface. Future studies to minimize this effect could use the *htrA*-NZ9000 strain, which may reduce protein proteolysis on the surface of *Lactococcus lactis* (Miyoshi *et al.*, 2002). HtrA is a surface protease of *Lactococcus lactis*, and inactivation of this protein was shown to reduce degradation of heterologous proteins expressed on the surface of *Lactococcus lactis*.

We tested the efficacy of our A2-expressing *Lactococcus lactis* live vaccines by immunizing mice subcutaneously and then challenging them with systemic infection with *Leishmania donovani*. We chose the subcutaneous route of administration because oral administration would most probably provide humoral mucosal immune responses, which should be non-protective against *Leishmania* infection. In addition, it was previously shown that parenteral injection of *Lactococcus lactis* live vaccine induced higher levels of antigen-specific immune responses in comparison with intragastric and intranasal vaccine

administration (Robinson *et al.*, 2004). We found that subcutaneous injection of $\sim 2 \times 10^9$ live bacteria in mice was well tolerated; however, occasionally there was formation of skin ulcerations at the site of injection, which were nevertheless resorbed during subsequent weeks. This is consistent with another study that safely administered 10^{10} live *Lactococcus lactis* to mice by subcutaneous injection (Buccato *et al.*, 2006).

Interestingly, immunization with only the cell-wall-anchored version of A2 in *Lactococcus lactis* (*Lactococcus lactis*/cwaA2) generated high levels of antibodies before challenge. Similar to our results, expression of the E7 antigen of human papilloma virus anchored to the cell wall also induced the highest levels of antigen-specific cellular immune responses following immunization of mice (Bermúdez-Humarán *et al.*, 2004). The undetectable levels of antibodies generated by the *Lactococcus lactis* strains expressing cytoplasmic and secreted A2 could have resulted from the antigen being mainly intracellular and sequestered from the immune system. In addition, secreted A2, which is mostly present in the bacterial culture supernatant, was probably washed away during preparation of the live bacteria prior to immunization. These results clearly demonstrate that the subcellular location of antigen expression has a critical influence on the immune response generated.

We tested the efficacy of our A2-expressing *Lactococcus lactis* live vaccines against visceral *Leishmania donovani* infection. Mice that were immunized with PBS and *Lactococcus lactis*/vector exhibited similar levels of liver parasitaemia (LDU). Notably, mice immunized with *Lactococcus lactis*/cytoA2 demonstrated the highest reduction in LDU, which was seen at both low- and high-dose parasite challenges, whilst immunization with *Lactococcus lactis*/cwaA2 also led to a reduction in LDU following only the low-dose challenge.

Similar to before challenge, only mice that were immunized with *Lactococcus lactis*/cwaA2 produced significant levels of A2-specific antibodies after challenge. It was also noteworthy that, although *Lactococcus lactis*/cwaA2 induced high levels of antigen-specific antibodies, it did not result in a stronger reduction in parasitaemia than *Lactococcus lactis*/cytoA2, as seen after high-dose infection with *Leishmania donovani*. Moreover, investigation of antigen-specific antibody isotypes (IgG₁ and IgG_{2a}) indicated that, following parasite challenge, there was further development of a Th2 immune response. This is similar to the Th2-biased response towards the antigen observed with a live *Lactococcus lactis* vaccine that expressed tetanus toxin fragment C (Robinson *et al.*, 2004). In addition, we demonstrated previously that *Lactococcus lactis* can induce dendritic cells to produce high levels of IL-10 *in vitro* (Yam *et al.*, 2008). Therefore, *Lactococcus lactis* may exhibit an ability to promote Th2-type immune responses. This would account for the lack of significant protection observed against *Leishmania* infection, which requires Th1-type cellular immune responses for protection. In contrast, other studies have shown that *Lactococcus lactis*-based live vaccines can also promote

balanced Th1/Th2-type immune responses (Hanniffy *et al.*, 2007; Lee *et al.*, 2009). As the production of IFN- γ and the development of Th1-type immune responses are known to be required for the clearance of intracellular pathogens such as *Leishmania*, we could consider enhancing the antigen-specific IFN- γ response and/or decreasing or neutralizing the IL-10 response. We are currently investigating the use of *Lactococcus lactis* co-expressing IL-12 and antigen to stimulate the production of antigen-specific IFN- γ .

The antigen-specific responses following immunization with A2-expressing *Lactococcus lactis* live vaccines may be improved by increasing the production, or increasing the size, of A2 in *Lactococcus lactis*. A2 is detected in *Leishmania donovani* as a family of proteins that range in size from 42 to 100 kDa, which corresponds to 40 to >90 repeats of the 10 aa motif (Ghedin *et al.*, 1997; Zhang & Matlashewski, 2001). Therefore, the wild-type A2 protein is much larger than the truncated A2 protein we generated, which contained ten copies of the 10 aa motif. Although the A2 epitopes of the wild-type and truncated protein are exactly the same, in the context of our *Lactococcus lactis* live vaccines, we may need to increase the protein size or enhance expression levels in order to augment the level of protection obtained against leishmaniasis in mice. Higher doses of antigen generally induce better antigen-specific immune responses (Grangette *et al.*, 2004).

In summary, we have shown that it is possible to express the *Leishmania* A2 antigen at different subcellular locations of the *Lactococcus lactis* bacterium and that the expression strategy has a major influence on the type of immune response generated against this cargo antigen. The *Lactococcus lactis* strains generated in this study provide an excellent foundation for further studies on live bacterial vaccines against leishmaniasis and other pathogens.

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