HOST RESPONSE TO INFECTION

Molecular and immunological characterisation of recombinant *Brucella abortus* glyceraldehyde-3-phosphate-dehydrogenase, a T- and B-cell reactive protein that induces partial protection when co-administered with an interleukin-12-expressing plasmid in a DNA vaccine formulation

GRACIA M. S. ROSINHA, ANDERSON MYIOSHI*, VASCO AZEVEDO*, GARY A. SPLITTER† and SERGIO C. OLIVEIRA

Department of Biochemistry and Immunology and Institute for Investigation in Immunology – Millenium Institute, and *Department of General Biology, Federal University of Minas Gerais, Belo Horizonte, Brazil and †Department of Animal Health and Biomedical Sciences, University of Wisconsin, Madison, WI 53706, USA

To identify antigens of *Brucella* spp. that are potentially involved in stimulating a protective T-cell-mediated immune response, previous studies identified 10 clones from a *Brucella abortus* 2308 genomic library with primed lymphocytes as probes. One selected positive clone (182) contained an insert of 1.2 kb which was identified, sequenced and characterised. The deduced amino acid sequence of the open reading frame (ORF) revealed 82% and 81% identity to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes from *Agrobacterium tumefaciens* and *Xanthobacter flavus*, respectively. Southern blot analysis demonstrated that the *gap* gene is present in only one copy in the *Brucella* genome. *B. abortus* GAPDH was then expressed in *Escherichia coli* as a fusion protein with the maltose-binding protein (MBP). To demonstrate the functional activity of *Brucella* GAPDH, *E. coli gap* mutants were transformed with a *Brucella* pMAL-*gap* construct. Genetic complementation was achieved and as a result *E. coli* mutants were able to grow on glucose or other carbon source medium. The humoral and cellular immune responses to the recombinant (r) GAPDH were characterised. In Western blots, sera from naturally infected cattle and sheep showed antibody reactivity against rGAPDH. In response to in-vitro stimulation by rGAPDH, splenocytes from mice vaccinated with rGAPDH or *B. abortus* S19 were able to produce γ -interferon and tumour necrosis factor- α but not interleukin (IL)-4. Furthermore, *gap* associated with murine *IL-12* gene in a DNA vaccine formulation partially protected mice against experimental infection.

Introduction

Brucella abortus is a facultative intracellular bacterium that infects man and domestic animals [1]. The pathological manifestations of brucellosis are diverse and include arthritis, endocarditis and meningitis in man, whereas bovine brucellosis is characterised by spontaneous abortion [2]. Pathogenic intracellular bacteria survive within a host cell by several mechanisms, circumventing a potent immune response [3]. Moreover,

little is known about the cellular and molecular mechanisms used by *B. abortus* to penetrate and resist destruction within host cells. Several lines of evidence suggest that cell-mediated immunity can limit the pathogenesis of brucellosis [4–6]. Studies in this laboratory have demonstrated that the induction of a Th1-type of immune response with production of γ -interferon (IFN- γ) and generation of cytotoxic CD8⁺ T cells appear to play a pivotal role in protection against *B. abortus* infection [7, 8]. *Brucella* proteins that induce an appropriate immune response are most likely responsible for generating host protection. Therefore, identifying these bacterial proteins would be critical for understanding the immune response to *B. abortus* infection and vaccination.

Received 25 Feb. 2002; accepted 14 March 2002.
Corresponding author: Dr S. C. Oliveira (e-mail: scozeus@icb.ufmg.br).

Several *Brucella* genes have been cloned, and the proteins that they encode have been characterised [9–13]. However, little is understood regarding the importance of these bacterial proteins in the immune response. Only few specific *Brucella* proteins such as L7/L12 ribosomal protein [14], peptides comprising certain epitopes of Cu/Zn superoxide dismutase [15] and P39 (a periplasmic binding protein) combined with CpG motifs [16] were able to engender partial protection based on T-cell responses. Therefore, a greater understanding of the relationship between *Brucella* proteins and host protection is required. Thus, the identification of individual bacterial proteins that trigger T lymphocytes is crucial to define how the immune system functions in this disease.

In an attempt to identify new *B. abortus* proteins capable of stimulating T cells, a *B. abortus* genomic library was screened with bovine lymphocytes from animals primed to the living bacterium [17]. The bacterial proteins from 10 clones of the 300 examined were recognised by primed lymphocytes. One of these clones, termed 182, was analysed in this study. DNA sequence analysis of clone 182 revealed the existence of an open reading frame (ORF) containing the whole sequence of *B. abortus gap* gene encoding the glyceraldehyde-3-phosphate-dehydrogenase enzyme (GAPDH). Interestingly, the GAPDH protein homologue has been associated with pathogenesis in other infectious disease models and has been shown to be a putative vaccine candidate against *Schistosoma japonicum* and *S. mansoni* infections [18, 19]. In the present study, the heterologous complementation of *Escherichia coli gap* mutant strains by *Brucella* GAPDH biological activity, was examined, and purified GAPDH recombinant (r) protein was produced for molecular and immunological characterisation. Furthermore, the study examined the recognition of rGAPDH protein by antibodies and Th1 cells from infected animals and the protection induced when it was administered as a DNA vaccine associated with *IL-12* gene.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *E. coli* DH5 α strain was grown at 37°C on Luria-Bertani medium [22]. All *E. coli* mutants

(DS111, DS112 and DS 113) and DS 110 were grown at 37°C on M63 medium supplemented with casamino acids 0.1%, 0.12 mM tryptophane, 0.17 mM methionine, 34 mM succinate and glycerol 0.88% or, in complementation studies, with 10 mM glucose and 10 mM maltose. Isopropyl- β -D-thiogalactoside (IPTG) was used at a final concentration of 25 mM in complementation experiments. *B. abortus* strains were grown in Brucella Broth (BB) (Difco Laboratories, Detroit, MI, USA). The antibiotic concentrations used were as follows: ampicillin 100 mg/L, erythromycin 200 mg/L and chloramphenicol 25 mg/L.

DNA procedures

DNA manipulation (i.e., preparation, digestion and ligation of plasmid DNA) and gel electrophoresis were performed by standard procedures [22].

DNA and amino acid sequence analysis

A 1.2-kb clone was obtained by screening a *B. abortus* 2308 genomic library with bovine lymphocytes from primed animals as probes [17]. Double-stranded DNA sequences of the 1.2-kb fragment inserted into the pBluescript II SK⁻ phagemid was performed by the dideoxy-chain termination method [23], with an Automated Laser Fluorescence DNA sequencer from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The clone was sequenced with the Thermo Sequenase kit and the primers used were M13 reverse or M13 universal sequence from Amersham Pharmacia Biotech. The sequence data were compiled and analysed with the sequence analysis program DNASIS V5.00 (Hitachi Software). Subsequent homology searches were performed with the BLAST programs available from the National Biotechnology Information Center [24]. Amino acid sequences were aligned with the ClustalW Service from the European Bioinformatics Institute available on the internet [<http://www2.ebi.ac.uk/clustalw>].

Southern blot analysis

The *Brucella* S19 strain was cultured for 72 h in BB. Then, 200 μ l of the bacterial suspension were harvested by centrifugation at 13 000 rpm for 5 min and the pellet was suspended in 1000 μ l of cell resuspension solution (10 mM Tris-HCl, pH 7.0, 10 mM EDTA,

Table 1. *E. coli* strains used

<i>E. coli</i> strains	Genotype and comments*	Reference or source
DH5 α	F ϕ 80dlacZ Δ M15 Δ (lacZYA ⁻ argF)U169 deoR recA1 endA1 hsdR17(rk ⁻ , mk ⁺) phoA supE44 λ ⁻ thi-1 gyrA96 relA1	Gibco BRL
DS 110	MG 1655 (K-12, F ⁻ λ ⁻)	20
DS 111	MG 1655 Δ gap B :: Erm	21
DS 112	MG 1655 Δ gap A :: Cm	21
DS 113	MG 1655 Δ gap A :: Cm Δ gap B :: Erm	21

*Erm and Cm indicate resistance conferred to erythromycin and chloramphenicol, respectively.

pH 8.0, 300 mM NaCl) and centrifuged. The pellet was resuspended in 300 μ l of cell lysis solution (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 10 mM NaCl), 300 μ l of lysozyme (10 mg/ml) were added and incubated at 65°C for 20 min. The lysed cell suspension was treated once with an equal volume of phenol-chloroform (v/v) and centrifuged. Then, 0.6 ml of chloroform was added to the aqueous phase recovered from the mixture and centrifuged again. Finally, the DNA was precipitated on 2.5 volumes of ice-cold ethanol (99.5%) plus 10 μ l of 5 M NaCl. The pellet was dissolved in 50 μ l of TE buffer (10 mM Tris-HCl, pH 7.2, 1 mM EDTA) and treated with RNAase 10 μ g/ml at 37°C for 15 min. The concentration of DNA was measured spectrophotometrically at 260 nm. For DNA hybridisation, 10 μ g of genomic DNA were digested with *Cla*I, *Eco*RI or *Hind*III. The resulting fragments were separated by agarose 1% gel electrophoresis, passively transferred on to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech) and UV-cross-linked. The *Eco*RI-*Hind*III fragment corresponding to the coding region of the *gap* gene was used as a DNA probe. This fragment was labelled with [α -³²P] dCTP by random priming (Gibco BRL). The hybridisation was performed under stringent conditions (38°C, formamide 50%) as described previously [22]. The nylon membrane was subjected to autoradiography for 18 h at -80°C.

Subcloning and expression of *Brucella gap* gene in *E. coli*

The *B. abortus gap* gene was amplified by PCR from the 1.2-kb genomic clone and subcloned into the expression vector pMAL-c2 (New England Biolabs, Beverly, MA, USA). In this vector, the cloned foreign gene was expressed as a fusion protein with maltose-binding protein (MBP) at the amino terminus so that the recombinant protein could be purified by affinity chromatography with an amylose resin. Primers, containing one artificial restriction site at each end, were constructed according to the *gap* nucleotide sequence. The primers sequences were (forward) 5'-GGGGAATT CATGGCAGTTTCGCGTCGCA-3' (*Eco*RI) and (reverse) 5'-CGCAAGCTTTCAGATCAGCTTGCCCAA-3' (*Hind*III). PCR was performed with a 10- μ l volume containing assay buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 500 mM MgCl₂), a mixture of the four deoxynucleoside triphosphates at 10 mM each, a 5 pmoles/ μ l concentration of each primer, 10 ng of template DNA and 2.5 U of *Taq* DNA polymerase (Promega). Amplification was performed with a PTC-100™ Programmable Thermal Controller (MJ Research) at 95°C for 3 min followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 45 s at 58°C and extension for 1 min at 72°C. The amplified gene fragment was digested with appropriate restriction endonucleases and cloned into pMAL-c2. The recombinant plasmid (pMAL-*gap*) was used to transform *E. coli* DH5 α , and single recombinant colonies were

selected. Plasmid DNA was extracted with Wizard Plus SV Minipreps (Promega) and digested with *Eco*RI and *Hind*III to verify the presence of the insert. The recombinant protein was expressed and purified as described previously [25]. The expression of the fusion protein resulted in the formation of the inclusion bodies that were solubilised by washing the bacterial pellet twice with wash buffer containing urea (0.1 M Tris-HCl, pH 8.8, and 5 M urea) and twice with water. To avoid degradation of the fusion protein, the following protease inhibitors were added during the extraction procedure: 10 mM EDTA, 2 mM PMSF, 1 μ M Pepstatin and 10 μ M E-64. The protein concentration was determined by the Bradford assay [26] with bovine serum albumin as a standard.

Molecular modelling

The amino acid sequence of GAPDH (target sequence) from *B. abortus* was submitted to theoretical molecular modeling by the SWISS-MODEL computer program (Automated Protein Modeling Server) [27]. Initially, a search was made for GAPDH sequences of known three-dimensional structure and a pair-wise alignment was performed with the target sequence. *Thermotoga maritima* and *Bacillus stearothermophilus* presented 55.4% and 57.4% sequence identity with *B. abortus* GAPDH protein, respectively. This level of sequence identity is sufficient to infer a homologous relationship and, therefore, a similar three-dimensional structure that permits the construction of a molecular model by homology-modelling techniques. The model was assessed with the computer program RasMol [28].

Complementation assay

To verify the functional activity of the *B. abortus* GAPDH, *E. coli* mutants for *gapA* (Δ gapA), *gapB* (Δ gapB), or *gapA/B* (Δ gapA/B) genes were transformed with 0.5 μ g of the pMAL-*gap* plasmid. To check the functional complementation, the transformed cells were selected by their ability to grow on glucose or other carbon source medium supplemented with the appropriate antibiotics.

SDS-PAGE and immunoblotting

E. coli extracts expressing the MBP-GAPDH fusion protein, and purified MBP-GAPDH protein were separated on a denaturing polyacrylamide 10% gel by electrophoresis [29]. From the gel, the proteins were transferred to nitrocellulose membranes (BioRad) by Western blot as described previously [30]. The nitrocellulose membranes were blocked with skim milk 5% at 4°C overnight, and treated with rabbit anti-MBP serum (1 in 10 000) or sera from naturally *Brucella*-infected cattle (1 in 1000) or sheep (1 in 50) for 2 h at room temperature. After reaction with the primary antibody, the blots were washed three times with TBST (0.5 M NaCl-0.02 M Tris, pH 7.5, Tween 20 0.05%) and

incubated for 1 h at room temperature with anti-rabbit IgG-alkaline phosphatase conjugate (1 in 10 000) (Promega), rabbit anti-bovine IgG-peroxidase conjugate (1 in 10 000) (Sigma) or rabbit anti-sheep IgG-peroxidase conjugate (1 in 2000) (Southern Biotechnology). After three washes with TBST, the reaction was developed after incubation at room temperature with NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) for alkaline phosphatase or DAB (3,3-diaminobenzidine tetrahydrochloride) (Gibco BRL) for peroxidase conjugated-antibody.

Cytokine assay

Spleens of mice vaccinated with rGAPDH or *B. abortus* strain S19 were passed through steel mesh to obtain single-cell suspensions. Splenocytes were then isolated by density gradient centrifugation with Ficoll (Sigma). Cells were washed twice with sterile PBS then cultured in RPMI 1640 medium supplemented with penicillin G sodium 100 U/ml, streptomycin sulphate 100 µg/ml, amphotericin B 250 ng/ml and fetal bovine serum (FBS) 10%, then placed at 1×10^6 cells/well in 96-well tissue-culture plates. Murine splenocytes from vaccinated animals were stimulated with the rGAPDH protein at 50 µg/ml. Unstimulated splenocytes were used as a negative control and splenocytes stimulated with concanavalin A (ConA) 5 µg/ml were used as T-cell activating control. After 48 h at 37°C in air with CO₂ 5%, splenocyte culture supernates were tested for the presence of cytokines by the ELISA DuoSet kit (R & D Systems) to mouse interleukin (IL)-4, γ -interferon (IFN- γ) and tumour necrosis factor (TNF)- α .

DNA vaccine construction and preparation

The *B. abortus* *gap* gene was amplified by PCR and subcloned into the mammalian expression vector pCMV-link (a gift from Dr J. Ulmer from Chiron). This experiment was performed with the same set of primers containing an artificial restriction site at each end (*EcoRI-HindIII*) and with the same protocol as described above for cloning the *gap* gene into pMAL-c2. The pCMV-*gap* plasmid was sequenced to confirm the presence and orientation of the *gap* gene. The mouse IL-12-encoding plasmid pCI-IL-12 was kindly provided by Dr J. S. Harms (University of Wisconsin-Madison, USA). The pCI-IL-12 plasmid was designed with a linker to fuse p35 cDNA with p40 cDNA to produce a single-chain IL-12 protein, ensuring not only that p35 and p40 were expressed equally, but also that no free p40 subunit interfered with IL-12 activity [31]. Both plasmids were prepared with a Wizard Maxiprep DNA purification kit (Promega, Madison, WI, USA) as described in the manufacturer's protocol.

Immunisation protocol

BALB/c mice, average 8 weeks old, were divided into five groups of eight mice each. For intramuscular DNA immunisation, mice were pre-injected with 10 mM cardiotoxin (Sigma) as described previously [32]. At weeks 0, 2, 4 and 6, 100 µg of pCMV-link (a gift from Dr J. Ulmer from Chiron), pCMV-*gap*, or pCMV-*gap* plus pCI-IL-12 constructs were administered intramuscularly into mouse quadriceps. As control groups, mice were immunised with 5×10^4 cfu of *B. abortus* strain 19 or PBS alone.

B. abortus challenge

Two weeks after the last immunisation, mice were challenged with 1×10^5 cfu of *B. abortus* strain 2308. Animals were then killed 2 weeks after challenge. Spleens from individual animals were homogenised in PBS, 10-fold serially diluted and plated on Brucella Broth Agar (Difco). Plates were incubated at 37°C in air with CO₂ 5% and the number of cfu was counted visually after 3 days.

Statistical analysis

Statistical analyses were performed with Student's *t* test in a computer software package (MINITAB; Minitab Inc., State College, PA, USA).

Nucleotide sequence accession no.

The nucleotide sequence presented in this study has been deposited in the GenBank under accession no. AF095338.

Results

Clone 182 containing the *gap* gene from *B. abortus*

One complete open reading frame of 1008 bp was identified in a 1213-bp clone. The nucleotide (nt) and amino acid (aa) sequences of *Brucella* *gap* gene are shown in Fig. 1. The coding region was translated with the computer program DNASIS v 5.0. The *Brucella* *gap* gene encodes a polypeptide of 336 aa with a predicted molecular mass of 37 kDa. The putative active site of the enzyme is located between aa 151 and 157. This ORF was subjected to protein and nucleic acid homology searches by the Basic Local Alignment Search Tool (BLAST) service at the National Center for Biotechnology Information (NCBI). This revealed that the ORF encoding the *Brucella* GAPDH aa sequence has 48%, 81% and 82% identity with the GAPDH encoded by *gap* genes of *E. coli*, *Xanthobacter flavus* and *Agrobacterium tumefaciens*, respectively. The alignment of the *Brucella* GAPDH aa sequence with those of other micro-organisms showed the highest degree of identity with the GAPDH from *A.*

1	ATG	GCA	GTT	CGC	GTC	GCA	ATC	AAC	GGT	TTT	GGC	CGC	ATT	GCG	GTA	ACC	ATC	CTT	CGC	GCC
1	M	A	V	R	V	A	I	N	G	F	G	R	I	A	V	T	I	L	R	A
61	ATC	GTG	GAA	TCG	GGG	CGC	ACC	GAC	ATT	CAG	GTC	GTC	GCC	ATC	AAC	GAT	CTC	GGC	CCG	GTC
21	I	V	E	S	G	R	T	D	I	Q	V	V	A	I	N	D	L	G	P	V
121	GAA	ACC	AAC	GCA	CAT	CTT	CTG	CGT	TAT	GAC	AGC	GTT	CAT	GGC	CGT	TTC	CCC	AAG	GAA	GTG
41	E	T	N	A	H	L	L	R	Y	D	S	V	H	G	R	F	P	K	E	V
181	GAG	GTT	GCA	GGC	GAT	ACG	ATC	GAT	GTT	GGC	TAC	GGC	CCG	ATC	AAG	GTT	CAT	GCC	GTC	CGC
61	E	V	A	G	D	T	I	D	V	G	Y	G	P	I	K	V	H	A	V	R
241	AAC	CCG	GCC	GAA	CTG	CCG	TGG	AAG	GAA	GAA	AAC	GTC	GAT	ATC	GCC	CTT	GAA	TGC	ACC	GGC
81	N	P	A	E	L	P	W	K	E	E	N	V	D	I	A	L	E	C	T	G
301	ATT	TTC	ACC	TCG	CGC	GAC	AAG	GCA	GCA	CTT	CAT	CTT	GAA	GCT	GGC	GCC	AAG	CGC	GTC	ATC
101	I	F	T	S	R	D	K	A	A	L	H	L	E	A	G	A	K	R	V	I
361	GTC	TCC	GCT	CCC	GCA	GAC	GGT	GCC	GAT	CTC	ACC	GTC	GTC	TAT	GGT	GTC	AAC	AAC	GAC	AAG
121	V	S	A	P	A	D	G	A	D	L	T	V	V	Y	G	V	N	N	D	K
421	CTG	ACG	AAG	GAC	CAT	CTG	GTC	ATC	TCC	AAC	GCT	TCG	TGT	ACC	ACC	AAC	TGC	CTT	GCG	CCG
141	L	T	K	D	H	L	V	I	S	N	<u>A</u>	<u>S</u>	<u>C</u>	<u>T</u>	<u>T</u>	<u>N</u>	<u>C</u>	L	A	P
481	GTG	GCT	CAG	GTT	CTC	AAC	GAT	ACT	ATC	GGT	ATC	GAA	AAG	GGC	TTC	ATG	ACC	ACG	ATC	CAC
161	V	A	Q	V	L	N	D	T	I	G	I	E	K	G	F	M	T	T	I	H
541	TCC	TAT	ACG	GGC	GAC	CAG	CCG	ACG	CTG	GAC	ACC	ATG	CAC	AAG	GAT	CTC	TAC	CGC	GCC	CGC
181	S	Y	T	G	D	Q	P	T	L	D	T	M	H	K	D	L	Y	R	A	R
601	GCC	GCT	GCC	CTT	TCC	ATG	ATC	CCG	ACC	TCG	ACG	GGT	GCG	GCC	AAG	GCC	GTC	GGT	CTC	GTT
201	A	A	A	L	S	M	I	P	T	S	T	G	A	A	K	A	V	G	L	V
661	CTG	CCG	GAA	CTG	AAA	GGC	AAG	CTC	GAC	GGC	GTT	GCA	ATT	CGC	GTC	CCG	ACC	CCA	AAT	GTC
221	L	P	E	L	K	G	K	L	D	G	V	A	I	R	V	P	T	P	N	V
721	TCG	GTC	GTT	GAT	CTC	ACC	TTC	ATC	GCA	AAG	CGT	GAA	ACC	ACC	GTT	GAA	GAA	GTC	AAC	AAT
241	S	V	V	D	L	T	F	I	A	K	R	E	T	T	V	E	E	V	N	N
781	GCG	ATC	AGC	GAA	GCC	GCC	AAT	GGC	CGC	CTC	AAG	GGC	ATT	CTC	GGC	TAT	ACC	GAT	GAG	AAG
261	A	I	S	E	A	A	N	G	R	L	K	G	I	L	G	Y	T	D	E	K
841	CTC	GTC	TCG	CAC	GAC	TTC	AAC	CAC	GAT	TCC	CAT	TCC	TCG	GTC	TTC	CAC	ACC	GAC	CAG	ACC
281	L	V	S	H	D	F	N	H	D	S	H	S	S	V	F	H	T	D	Q	T
901	AAG	GTT	ATG	GAA	GGC	ACC	ATG	GTG	CGT	ATC	CTG	TCG	TGG	TAC	GAC	AAT	GAA	TGG	GGC	TTC
301	K	V	M	E	G	T	M	V	R	I	L	S	W	Y	D	N	E	W	G	F
961	TCC	AGC	CGC	ATG	CGC	GAC	ACC	GCC	GTC	GCT	TTG	GGC	AAG	CTG	ATC	TGA				
321	S	S	R	M	R	D	T	A	V	A	L	G	K	L	I	*				

Fig. 1. The nucleotide and deduced aa sequence of the *B. abortus gap* gene. The putative active site is underlined. The asterisk denotes the termination codon.

tumefaciens, which is closer phylogenetically to the genus *Brucella* (Fig. 2).

gap gene copy number

The *gap* gene appears to be a single copy gene in the *Brucella* genome. Southern blotting of the genomic DNA with a fragment comprising the *gap* ORF as probe showed a single band in lanes 3 and 4 that contained DNA digested with *Eco*RI or *Hind*III (both enzymes do not cut within the coding region of GAPDH) and two bands in lane 2 that has DNA digested with *Cl*al (this enzyme has one site within the coding region of GAPDH) (Fig. 3).

Brucella GAPDH three-dimensional structure

The model constructed for *B. abortus* GAPDH showed

that this molecule adopts the same three-dimensional topology as GAPDH of other micro-organisms. This structure consists of 260 H-bridges, 14 α -helices, 24 β -sheets and 37 loops (Fig. 4). This model suggests a glycolytic function for *Brucella* GAPDH similar to other bacterial GAPDH, based upon its tertiary structure. The enzyme active site, composed of alanine, serine, cysteine, threonine, threonine, asparagine and cysteine residues, is shown in ball-and-stick format.

Functional complementation of *E. coli* mutants by *B. abortus gap* gene

To determine whether the *gap* gene sequence of *B. abortus* encodes a functional GAPDH, complementation of three *E. coli* mutants was tested. The plasmid pMAL-*gap*, containing all translational signals thought to be necessary for expression of *Brucella* GAPDH

Ba	MAVRVAINGFGRIVAVTILRAIVESGRTDIQVVAINDLGPVETNAHLLRYDSVHGRFPKEV	60
Xf	MSVKVAINGFGRIGRNVLRRAIESGRTDIEVVVAINDLGPVETNAHLRFDSVHGRFPGEV	60
At	MTVKVAINGFGRIGRNVLRRAIVESGRTDIEVVVAINDLGPVETNAHLLRYDSIHGRFPADV	60
Sm	MTVKVAINGFGRIGRNVLRRAIVESGRTDIEVVVAINDLGPVETNAHLLRFDSIHGRFPADV	60
Ec	MTIKVGINFGRIGRIVFRAAQK--RSDIEIVVAINDLLDADYMLKYDSTHGRFDGTV	58
	:::.*****. :*: * : *:::***** .: *:::*** **:* *	
Ba	EVAGDTIDVGYG-PIKVHAVRNPAELPWKEENVDALECTGIFTSRDKAALHLEAGAKRV	119
Xf	KVAGDTIDVGRG-PIKVTAVRNPAELPHKELGVDIALECTGIFTSRDKAAAHLAAGAKRV	119
At	KVAGDTIIVGGGKPIKVTAVRDPATLPHKELGVDIALECTGIFTARDKAAAHLTAGAKRV	120
Sm	KVDGDAIVNNGKPIKVTAINRPAELPHKELGVDIAMECTGIFTARDKAAAHLEAGAKRV	120
Ec	EVKDGHLIVNGK-KIRVTAERDPANLKWDEVGVVVAEATGLFLTDETARKHITAGAKKV	117
	:* .. : :. *:* * *:* * . * .*: . *.*:* : :.* *: *****:	
Ba	IVSAPADGADLTVVYGVNNDKLTDKHLVISNASCTTNCLAPVAQVLNDTIGIEKGFMTTI	179
Xf	LVSAPADGADLTVVYGVNHEKLTNEHLVVSNASCTTNCLAPVAKVLNDAVGIEKGFMTTI	179
At	IVSAPADGADLTVVFGVNDDQLTKDHLVISNASCTTNCLVPVVKVLDVIGIDHGFMTTI	180
Sm	IVSAPADGADLTVVYGVNHDKLTDKHLVISNASCTTNCLVPVAKVLHDAVGIDHGMFTTI	180
Ec	VMTGPSKDNTPMFVKGANFDKYAG-QDIVSNASCTTNCLAPLAKVINDNFGIIEGLMTTV	176
	:::.*::.. . * *.* : : : : :*****.*:::*. * .** .*:***:	
Ba	HSYTGDAQPTLD-TMHKDLRYRARAALSMIPTSTGAAKAVGLVLPKLGKLDGVAIRVPTP	238
Xf	HAYTGDAQPTLD-TMHKDLRYRARAALSMIPTSTGAAKAVGLVLPKLGKLDGTSIRVPTP	238
At	HSYTGDAQPTLD-TMHKDLRYRARAALSMIPTSTGAAKAVGLVLPKLGKLDGTSIRVPTP	239
Sm	HSYTNDQPSLD-QMHKDLRYRARAALSMIPTSTGAAKAVGLVLPKLGKLDGTSIRVPTP	239
Ec	HATTATQKTVDGFSHKDWRGGRGASQNIIPSSTGAAKAVGKVLPELNGKLTGMAFRVPTP	236
	*: * * ::* *** .*: .:***:***** ***.* *** * :.*****	
Ba	NVSVDLTFIAKRETTVEEVNNAISEAANGRLKGIILGYTDEKLVSHDFNHDSSHVSFHTD	298
Xf	NVSVIDLKFVAKRATSKEEINEAIIAASQQLKGIILGFTDQPNVSIIDFNHNPNSSTFHL	298
At	NVSVDLKFIAKRDTTVEEVNEAIIKASNGKLKGIILGYTEEPLVSRDFNHDSSHSSIFAID	299
Sm	NVSVDLKFVAKRETTKEEINAAIKAADGPLKGVLYTLAPNVSIDFNHDPRSSVFHMD	299
Ec	NVSVDLTVRLEKAATYEQIKAADVAAAEGEMKGVLYTEDDVVSTDFNGEVCTSVFDAK	296
	*****:~::.. : : : *::: *; *.. :*:***:* ** *** : : * * .	
Ba	QTKVMEGTMVRILSWYDNEWGFSSRMRTAVALGKLI	335
Xf	QTKVMEGTLVRVLSWYDNEWGFSSNRMSDTAVAMGKLG	335 (81%)
At	QTKVMEGNFVRVLSWYDNEWGFSSNRMSDTAVAFAKTI	336 (82%)
Sm	QTKVMEGKFVLSWYDNEWGFSSNRMSDTAVALSKLI	336 (80%)
Ec	AGIALNDNFVKLVSWYDNETGYSNKVLDLIAHISK--	331 (48%)
	.:~:::~* :***** *~::: * . :.*	

Fig. 2. Multiple alignment of GAPDH aa sequences from *B. abortus* (Ba), *Xanthobacter flavus* (Xf), *Agrobacterium tumefaciens* (At), *Sinorhizobium meliloti* (Sm) and *E. coli* (Ec). Gaps are indicated by dashes. (*) identical residues, (:) conserved substitutions, (.) semi-conserved substitutions. The percentages of identity with the aa sequence of GAPDH from *B. abortus* are indicated in parentheses.

protein, was used to transform *E. coli gap* mutants unable to grow on glucose minimal medium. As shown in Table 2, growth of *E. coli* strains DS110 and DS111 was not affected in the presence of glucose or maltose. In contrast, no growth was detected when *E. coli* mutants DS112 and DS113 were used. However, after complementation, DS112 and DS113 mutant strains harbouring the pMAL-*gap* plasmid grew in the presence of glucose or maltose, indicating functional activity as a glycolytic enzyme of the GAPDH from *B. abortus*.

Recognition of *Brucella rGAPDH* by sera of infected animals

The heterologous expression of *Brucella* GAPDH protein was performed in *E. coli* as an MBP fusion. Recombinant GAPDH was purified by affinity chromatography on an amylose resin column as demonstrated previously [25]. The *E. coli* extract carrying the

pMAL-*gap* construct and the purified rGAPDH were analysed by immunoblotting with rabbit antiserum to MBP and sera from naturally infected cattle and sheep (Fig. 5). The observed molecular mass of the recombinant protein was in agreement with the estimated molecular mass of the MBP fusion, 79 kDa (37 kDa for GAPDH and 42 kDa for MBP). The immunoblotting analysis confirmed the identity of the MBP-GAPDH fusion protein produced by rabbit anti-MBP antibody recognition. Lastly, this result demonstrated that IgG antibodies to rGAPDH were detected in sera of naturally infected cattle and sheep. The detection of total IgG specific for rGAPDH in sera of these animals is relevant because they are the natural hosts for *Brucella* infection.

Induction of a Th1-type of immune response by rGAPDH immunisation

To further examine T-cell reactivity to *Brucella*

Fig. 3. Southern blot analysis of genomic DNA from *B. abortus*. DNA undigested (lane 1), digested with *Cla*I (2), *Eco*RI (3) or *Hind*III (4) was subjected to agarose 1% gel electrophoresis for 5 h at 100 V and passively transferred on to a nylon membrane, Hybond-N. The hybridisation was performed with the 1008-bp fragment containing the *Brucella gap* gene.

Fig. 4. Ribbon diagram of the theoretical molecular model of the *B. abortus* GAPDH enzyme. Residues shown in a ball-and-stick format represent the active site of the enzyme. This model was constructed with the SWISS-MODEL program based on the three-dimensional structure of homologous molecules from *Thermotoga maritima* and *Bacillus stearothermophilus*. The figure was visualised with the computer program RasMol.

rGAPDH, the cytokine profile of mice immunised with rGAPDH or *B. abortus* S19 was determined. Fig. 6 shows that high levels of IFN- γ and TNF- α were detected in cell supernates of both groups of mice that had been stimulated *in vitro* with rGAPDH. Animals immunised with rGAPDH showed a slight increase in the production of IL-4 compared with mice vaccinated with strain S19; however, this was not statistically significant. As negative control, MBP alone was used to stimulate splenocytes, and no significant T-cell activation was detected in either mouse group (data not shown). In contrast, ConA significantly stimulated cytokine production by splenocytes from both vaccinated mouse groups (data not shown). Nevertheless, groups immunised with both rGAPDH and strain S19 demonstrated a polarised Th1-type of immune response. Furthermore, these results confirmed that T cells from mice vaccinated with strain S19 could recognise rGAPDH, as previously shown when lymphocytes from primed cattle were used as probes to screen a *Brucella* genomic library [17].

Protection efficacy of gap DNA immunisation

Two weeks after the last DNA immunisation, mice were challenged with 1×10^5 cfu of *B. abortus* strain 2308. The protective immunity was determined 2 weeks after infection by determining the number of *B. abortus* cfu in the spleen. *gap* gene alone engendered no protection against challenge with a *B. abortus* virulent strain, whereas *gap* associated with murine IL-12 gene as adjuvant induced partial protection against infection (Table 3).

Discussion

The search for new vaccines against *Brucella* spp., overcoming the drawbacks of current live attenuated vaccines, is a goal of several laboratories world-wide. Efforts have been made to isolate, identify and characterise new bacterial antigens. However, only moderate protection has been induced by these subunit vaccines [14–16]. One of the more promising candidates was identified in this laboratory, the *Brucella* L7/L12 ribosomal protein, a T-cell immunodominant antigen [14]. Taking a different approach, a genomic library of *B. abortus* 2308 was screened for expressed antigens that reacted with T lymphocytes from cattle vaccinated with *B. abortus* S19 [17]. Clone 182 was one of 10 positive clones isolated from the library. The deduced aa sequence of the ORF from clone 182 demonstrated homology to GAPDH of different bacterial species, mainly with GAPDH of *A. tumefaciens*, where 82% sequence identity was found. Furthermore, a molecular model to predict the three-dimensional structure of *Brucella* GAPDH was performed, and its topology consisting of 260 H-bridges, 14 α -helices, 24 β -sheets and 37 loops was very similar to tertiary structure of the GAPDH from other bacteria. However,

Table 2. Complementation analysis of *E. coli* mutant strains with *B. abortus gap* gene

Strain no.	Growth on M63 medium supplemented with			
	glucose	glycerol	maltose	succinate + glycerol
DS 110	+	+	+	+
DS 111 Δ gapB	+	+	+	+
DS 112 Δ gapA	—	+	—	+
DS 113 Δ gapA Δ gapB	—	+	—	+
DS 111 Δ gapB (pMAL-gap)	+	+	+	+
DS 112 Δ gapA (pMAL-gap)	+	+	+	+
DS 113 Δ gapA Δ gapB (pMAL-gap)	+	+	+	+

+, growth; —, no detectable growth.

Fig. 5. (A) Coomassie Blue-stained SDS-10% PAGE profile of *E. coli* extracts expressing the MBP-GAPDH fusion protein (lane 1), or purified MBP-GAPDH fusion protein (2). (B) Western blot analysis showing MBP-GAPDH recognition by rabbit anti-MBP antibodies (lane 3) and sera from cattle (4) and sheep (5), both naturally infected with *Brucella*. The arrow indicates the MBP-GAPDH band. The numbers on the left indicate the mol. wt standards (kDa) (Amersham Pharmacia Biotech).

to determine the biological activity of *Brucella* GAPDH *E. coli gap A* (DS112), *gap B* (DS111) or the null (DS113) mutant (which are unable to grow on glucose minimal medium) were complemented with the pMAL-*gap* construct. *E. coli* mutant strains DS112 and DS113 complemented with the pMAL-*gap* plasmid grew in the presence of glucose or maltose, demonstrating the biological function of the GAPDH from *B. abortus* as a glycolytic enzyme.

GAPDH is a glycolytic enzyme; however, a number of other physiological functions have been associated with this molecule – such as assembly of microtubules [33], adhesion to fibronectin, myosin and actin [34], and

the ability to serve as a receptor for plasmin [35]. Interestingly, immunological studies of anti-*S. mansoni* responses in individuals from endemic areas identified GAPDH (Sm37) as a vaccine candidate based on the correlation of antibody titres induced against this antigen and resistance to re-infection [36]. Also, GAPDH has been involved as an important immunogenic molecule in several infectious disease models such as African trypanosomiasis, candidiasis, Lyme disease and Chagas' diseases [37–40].

This study analysed both the humoral and the cell-mediated immune responses induced by *Brucella* rGAPDH. Immunoblotting analysis revealed that IgG

Fig. 6. Cytokine responses from splenocytes derived from rGAPDH or *B. abortus* S19-primed BALB/c mice. The cells were stimulated *in vitro* with rGAPDH 50 µg/ml or ConA (data not shown) for 48 h. Levels of IL-4 (□), IFN-γ (▨) or TNF-α (■) present in the cell culture supernates were assayed by ELISA. Bars indicate the mean values (SD) of cytokines (pg/ml) of four mice. Differences in relation to rGAPDH treatment are denoted by * for $p < 0.05$.

Table 3. Protective immunity induced by *gap* DNA immunisation

Treatment	Mean (SD) log ₁₀ cfu in mouse spleen	Log ₁₀ unit of protection
PBS	7.00 (0.25)	—
pCMV-link	6.78 (0.31)	—
pCMV-gap	7.00 (0.15)	—
pCI-IL-12	6.89 (0.34)	—
pCMV-gap + pCI-IL-12	6.21 (0.50)	0.68*
<i>B. abortus</i> S 19	4.50 (0.28)	2.50†

* $p < 0.05$, significant compared with pCI-IL-12 control group.

† $p < 0.05$, significant compared with PBS control group.

antibodies from naturally infected cattle and sheep recognised *Brucella* rGAPDH. The detection of specific antibodies to rGAPDH in sera from these hosts is relevant for several reasons. First, cattle and sheep are important natural hosts for *Brucella* infection [1]. Second, levels of IgG in infected hosts are correlated with the presence of active infection [41]. Third, protective antibodies in the mouse model appear to be predominantly of the IgG isotype [42]. However, because of the intracellular nature of *Brucella* infection, the induction of a cellular immune response is critical for effective host clearance of the bacterium. Therefore, IL-4, IFN-γ and TNF-α levels were measured in splenocyte supernates of mice vaccinated with rGAPDH or *B. abortus* S19. The results clearly indicated that rGAPDH induced a Th1-type of immune response in mice, although the levels of IFN-γ and TNF-α in the rGAPDH-immunised group were lower than in the group vaccinated with strain S19. A similar observation was reported by our group and others when a *Brucella* recombinant protein was used as antigen, and the less potent T-cell activation stimulus is probably due to the antigenic complexity of *B. abortus* S19 compared with a single molecule [43]. Immunisation of mice with strain S19 induces protective immunity and splenocytes from such mice produce high levels of IFN-γ. This fact is consistent with the

hypothesis that a protective immune response to *Brucella* infection requires the production of pro-inflammatory cytokines such as IFN-γ and TNF-α [44].

As GAPDH is a conserved protein, at least in the functionally constrained domains of the molecule, the *Brucella* GAPDH aa sequence was analysed with a TEPITOPE algorithm which predicts HLA-DR ligands within the repeated GAPDH motifs [45]. Comparison analysis of *Brucella* GAPDH aa sequence with human, mouse and cattle GAPDH indicated that this protein shares only 42%, 42% and 44% identity, respectively. TEPITOPE prediction revealed that the promiscuous T-cell epitopes are on aa positions 7–20, 70–80, 195–213 and 306–325 of *Brucella* GAPDH and, therefore, they are not overlapping the region of the molecule homologous to human, mouse or cattle GAPDH (data not shown). Furthermore, it was demonstrated that non-primed T cells from human, mouse or cattle were not activated following *in-vitro* stimulation with rGAPDH (data not shown). Similar findings were observed by Pitarch and co-workers with sera from healthy subjects to *Candida albicans* GAPDH [40]. Therefore, vaccination experiments with *Brucella* GAPDH would probably not induce cross-reactive recognition by human or animal T cells, thus avoiding autoimmune reactions.

Gene vaccines have been employed successfully in animal studies to induce protective immunity against a variety of bacteria, viruses and parasites [46]. This type of vaccine is capable of eliciting a strong cell-mediated immunity that is required to control infection by many intracellular agents [47]. Moreover, endogenous expression of antigen from DNA introduced into host cells leads to processed peptides presented with the major histocompatibility complex class I, able to induce cytotoxic T lymphocytes [48]. As CD8⁺ T cells seems to be the major T-cell subset involved in controlling *Brucella* infection [8], the *gap* gene was used in a DNA vaccine formulation to protect mice against experimental infection. The *gap* gene delivered intramuscularly induced no protection in BALB/c mice, whereas administration of pCMV-gap plus pCI-IL-12 engendered moderate protection against challenge. This synergic effect of IL-12 of *gap* DNA immunisation could be related to direct activation of CD8⁺ T cells or enhanced type I cytokine production, or both [49].

The results presented here demonstrate the involvement of rGAPDH in the immune response to *B. abortus*. rGAPDH was shown to be immunoreactive, inducing both humoral and cellular immune responses in natural hosts and mice during the course of infection. T-cell activation following rGAPDH *in-vitro* stimulation, measured here by IFN-γ and TNF-α, confirmed the T-cell reactivity of this molecule as originally observed from the genomic library [17]. Furthermore, DNA immunisation with *gap* gene plus murine IL-12-expressing plasmid DNA induced partial protection against *Brucella* infection.

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), PADCT/CNPq/CBAB, FAPEMIG and National Institutes of Health grant RO1 AI48490. We thank Dr Sophie Rahuel-Clermont from the Université Henri Poincaré Nancy I-France for providing the *E. coli* gap mutants.

References

- Nicoletti PL. Relationship between animal and human disease. In: Young EJ, Corbel MJ (eds) *Brucellosis: clinical and laboratory aspects*. Boca Raton, FL, CRC Press. 1989: 41–51.
- Young EJ. Human brucellosis. *Rev Infect Dis* 1983; **5**: 821–842.
- Kaufmann SHE. Immunity to intracellular bacteria. *Ann Rev Immunol* 1993; **11**: 129–163.
- Araya LN, Winter AJ. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. *Infect Immun* 1990; **58**: 254–256.
- Golding B, Scott DE, Scharf O *et al.* Immunity and protection against *Brucella abortus*. *Microbes Infect* 2001; **3**: 43–48.
- Zhan Y, Yang J, Cheers C. Cytokine response of T-cell subsets from *Brucella abortus* infected mice to soluble *Brucella* proteins. *Infect Immun* 1993; **61**: 2841–2847.
- Oliveira SC, Zhu Y, Splitter GA. Recombinant L7/L12 ribosomal protein and γ -irradiated *Brucella abortus* induce a T-helper 1 subset response from murine CD4⁺ T cells. *Immunology* 1994; **83**: 659–664.
- Oliveira SC, Splitter GA. CD8⁺ type 1 CD44hi CD45Rblo T lymphocytes control intracellular *Brucella abortus* infection as demonstrated in major histocompatibility complex class I- and Class II-deficient mice. *Eur J Immunol* 1995; **25**: 2551–2557.
- Chirhart-Gilleland RL, Kovach ME, Elzer PH, Jennings SR, Roop RM. Identification and characterization of a 14-kilodalton *Brucella abortus* protein reactive with antibodies from naturally and experimentally infected hosts and T lymphocytes from experimentally infected BALB/c mice. *Infect Immun* 1998; **66**: 4000–4003.
- Jubier-Maurin V, Boigegrain R-A, Cloeckert A *et al.* Major outer membrane protein Omp25 of *Brucella suis* is involved in inhibition of tumor necrosis factor alpha production during infection of human macrophages. *Infect Immun* 2001; **69**: 4823–4830.
- Lin J, Adams LG, Ficht TA. Immunological response to the *Brucella abortus* GroEL homolog. *Infect Immun* 1996; **64**: 4396–4400.
- Vemulapalli R, Duncan AJ, Boyle SM, Sriranganathan N, Toth TE, Schurig GG. Cloning and sequencing of yajC and secD homologs of *Brucella abortus* and demonstration of immune responses to YajC in mice vaccinated with *B. abortus* RB51. *Infect Immun* 1998; **66**: 5684–5691.
- Zygmunt MS, Diaz MA, Teixeira-Gomes AP, Cloeckert A. Cloning, nucleotide sequence, and expression of the *Brucella melitensis* *sucB* gene coding for an immunogenic dihydro-lipoamide succinyltransferase homologous protein. *Infect Immun* 2001; **69**: 6537–6540.
- Oliveira SC, Splitter GA. Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against *Brucella abortus* infection. *Vaccine* 1996; **14**: 959–962.
- Tabatabai LB, Pugh GW. Modulation of immune responses in Balb/c mice vaccinated with *Brucella abortus* Cu-Zn superoxide dismutase synthetic peptide vaccine. *Vaccine* 1994; **12**: 919–924.
- Al-Mariri A, Tibor A, Mertens P *et al.* Protection of BALB/c mice against *Brucella abortus* 544 challenge by vaccination with bacterioferritin or P39 recombinant proteins with CpG oligodeoxynucleotides as adjuvant. *Infect Immun* 2001; **69**: 4816–4822.
- Zhu Y, Oliveira SC, Splitter GA. Isolation of *Brucella abortus* *sbb* and *uvrA* genes from a genomic library by use of lymphocytes as probes. *Infect Immun* 1993; **61**: 5339–5344.
- Argiro LL, Kohlstädt SS, Henri S *et al.* Identification of a candidate vaccine peptide on the 37 kDa *Schistosoma mansoni* GAPDH. *Vaccine* 2000; **18**: 2039–2048.
- Waine GJ, Becker M, Yang W, Kalinna B, McManus DP. Cloning, molecular characterization, and functional activity of *Schistosoma japonicum* glyceraldehyde-3-phosphate dehydrogenase, a putative vaccine candidate against schistosomiasis japonica. *Infect Immun* 1993; **61**: 4716–4723.
- Bachmann BJ. Linkage map of *Escherichia coli* K-12, edition 7. *Microbiol Rev* 1983; **47**: 180–230.
- Seta FD, Boschi-Muller S, Vignais ML, Branlant G. Characterization of *Escherichia coli* strains with *gapA* and *gapB* genes deleted. *J Bacteriol* 1997; **179**: 5218–5221.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning. A laboratory manual*, 2nd edn. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press. 1989.
- Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977; **74**: 5463–5467.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; **215**: 403–410.
- Oliveira SC, Splitter GA. Subcloning and expression of the *Brucella abortus* L7/L12 ribosomal gene and T-lymphocyte recognition of the recombinant protein. *Infect Immun* 1994; **62**: 5201–5204.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
- Peitsch MC. ProMod and Swiss-Model: Internet-based tools for automated comparative protein modelling. *Biochem Soc Trans* 1996; **24**: 274–279.
- Sayle RA, Milner-White EJ. RASMOL: biomolecular graphics for all. *Trends Biochem Sci* 1995; **20**: 374–376.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350–4354.
- Lee Y-L, Tao M-H, Chow Y-H, Chiang B-L. Construction of vectors expressing bioactive heterodimeric and single-chain murine interleukin-12 for gene therapy. *Hum Gene Ther* 1998; **9**: 457–465.
- Leclercq S, Harms JS, Rosinha GMS, Azevedo V, Oliveira SC. Induction of a Th1-type of immune response but not protective immunity by intramuscular DNA immunisation with *Brucella abortus* GroEL heat-shock gene. *J Med Microbiol* 2002; **51**: 20–26.
- Huitorel P, Pantaloni D. Bundling of microtubules by glyceraldehyde-3-phosphate dehydrogenase and its modulation by ATP. *Eur J Biochem* 1985; **150**: 265–269.
- Pancholi V, Fischetti VA. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. *J Exp Med* 1992; **176**: 415–426.
- Lottenberg R, Broder CC, Boyle MDP. Identification of a specific receptor for plasmin on a group A *Streptococcus*. *Infect Immun* 1987; **55**: 1914–1918.
- Dessein AJ, Begley M, Demeure C *et al.* Human resistance to *Schistosoma mansoni* is associated with IgG reactivity to a 37-kDa larval surface antigen. *J Immunol* 1988; **140**: 2727–2736.
- Balaban N, Waitaha HK, Njogu AR, Goldman R. Intracellular antigens (microtubule-associated protein copurified with glycosomal enzymes) – possible vaccines against trypanosomiasis. *J Infect Dis* 1995; **172**: 845–850.
- Jungblut PR, Grabher G, Stoffler G. Comprehensive detection of immunorelevant *Borrelia garinii* antigens by two-dimensional electrophoresis. *Electrophoresis* 1999; **20**: 3611–3622.
- Grijalva MJ, Goodrum KJ, Rowland EC. Immunological characterization of antigens released by *Trypanosoma cruzi*-infected cells. *J Parasitol* 1999; **85**: 663–671.
- Pitarch A, Pardo M, Jimenez A. *et al.* Two-dimensional gel electrophoresis as analytical tool for identifying *Candida albicans* immunogenic proteins. *Electrophoresis* 1999; **20**: 1001–1010.
- Diaz R, Moriyón I. Laboratory techniques in the diagnosis of human brucellosis. In: Young EJ, Corbel MJ (eds) *Brucellosis: clinical and laboratory aspects*. Boca Raton, FL, CRC Press. 1989: 73–83.
- Elzer PH, Jacobson RH, Jones SM, Nielsen KH, Douglas JT, Winter AJ. Antibody-mediated protection against *Brucella abortus* in BALB/c mice at successive periods after infection: variation between virulent strain 2308 and attenuated strain 19. *Immunology* 1994; **82**: 651–658.

43. Oliveira SC, Harms JS, Banai M, Splitter GA. Recombinant *Brucella abortus* proteins that induce proliferation and gamma-interferon secretion by CD4+ T cells from Brucella-vaccinated mice and delayed-type hypersensitivity in sensitized guinea pigs. *Cell Immunol* 1996; **172**: 262–268.
44. Murphy EA, Sathiyaseelan J, Parent MA, Zou B, Baldwin CL. Interferon-gamma is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. *Immunology* 2001; **103**: 511–518.
45. de Lalla C, Sturniolo T, Abbruzzese L *et al.* Cutting edge: identification of novel T cell epitopes in Lol p5a by computational prediction. *J Immunol* 1999; **163**: 1725–1729.
46. Donnelly JJ, Ulmer JN, Shiver JW, Liu MA. DNA vaccines. *Annu Rev Immunol* 1997; **16**: 617–648.
47. Gurunathan S, Wu C-Y, Freidag BL, Seder RA. DNA vaccines: a key for inducing long-term cellular immunity. *Curr Opin Immunol* 2000; **12**: 442–447.
48. Corr M, Lee DJ, Carson DA, Tighe H. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J Exp Med* 1996; **184**: 1555–1560.
49. Park AY, Scott P. IL-12: keeping cell-mediated immunity alive. *Scand J Immunol* 2001; **53**: 529–532.