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A Bicistronic DNA Vaccine Containing Apical Membrane Antigen 1 and Merozoite Surface Protein 4/5 Can Prime Humoral and Cellular Immune Responses and Partially Protect Mice against Virulent *Plasmodium chabaudi adami* DS Malaria

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The ultimate malaria vaccine will require the delivery of multiple antigens from different stages of the complex malaria life cycle. In order to efficiently deliver multiple antigens with use of DNA vaccine technology, new antigen delivery systems must be assessed. This study utilized a bicistronic vector construct, containing an internal ribosome entry site, expressing a combination of malarial candidate antigens: merozoite surface protein 4/5 (MSP4/5) (fused to a monocyte chemotactic protein 3 chemoattractant sequence) and apical membrane antigen 1 (AMA-1) (fused to a tissue plasminogen activator secretion signal). Transfection of COS 7 cells with bicistronic plasmids resulted in production and secretion of both AMA-1 and MSP4/5 in vitro. Vaccination of BALB/c mice via intraepidermal gene gun and intramuscular routes against AMA-1 and MSP4/5 resulted in antibody production and significant in vitro proliferation of splenocytes stimulated by both AMA-1 and MSP4/5. Survival of BALB/c mice vaccinated with bicistronic constructs after lethal *Plasmodium chabaudi adami* DS erythrocytic-stage challenge was variable, although significant increases in survival and reductions in peak parasitemia were observed in several challenge trials when the vaccine was delivered by the intramuscular route. This study using a murine model demonstrates that the delivery of malarial antigens via bicistronic vectors is feasible. Further experimentation with bicistronic delivery systems is required for the optimization and refinement of DNA vaccines to effectively prime protective immune responses against malaria.

It is believed that the ultimate malaria vaccine will require the delivery of multiple antigens from different stages of the complex malaria life cycle (8, 17). Delivery of combinations of malarial antigens can evoke enhanced immune responses and protect to a greater extent than can a single antigen alone, as well as overcome genetic restrictions in different mouse strains (3, 9, 15, 16, 32).

Combinations of malarial antigens delivered as malarial DNA vaccines in primates have also resulted in enhanced levels of cytotoxic T lymphocytes to pre-erythrocytic-stage vaccines (32) and enhanced antibody responses to erythrocytic-stage malarial vaccines (15). It is believed that "first-generation" DNA vaccines (i.e., delivery of only a single plasmid-antigen DNA) are not optimal to protect against malaria and that immune enhancement strategies for DNA vaccination alone are required for this method of vaccination to be practical (reviewed in reference 8).

The use of multivalent DNA vaccine expression systems such as bicistronic vectors may enable more efficient delivery of antigen in malaria DNA vaccination and promote synergistic responses between malarial antigens. Testing of viral bicistronic and polycistronic vectors in cancer gene therapy has been widely used to obtain synergistic effects with use of combinations of antitumor genes (reviewed in reference 6). Examples of nonviral bicistronic vector use as DNA vaccines include vaccines against hepatitis B (5) and hepatitis C (4), as well as vaccination against B-cell lymphoma (28).

Bicistronic plasmids utilize an internal ribosome entry site (IRES) placed between two coding regions. This allows ribosomes to attach to mRNA and translate the downstream coding sequence, while the upstream sequence is translated by cap-dependent mechanisms (6). IRES sequences have been found in viral and eukaryotic mRNA, all differing in primary sequence, nucleotide length, and secondary structure, although they do share a hairpin nucleotide structure promoting small ribosomal subunit binding (reviewed in reference 22). The nucleotide composition of genes flanking the IRES is also an important factor in the expression of the genes contained within bicistronic vectors, both in vivo and in vitro (6, 12).

Bicistronic delivery of malarial DNA vaccines may have the potential to enhance the ability of first-generation DNA vaccines to prime an immune response prior to a malaria infection. In this study, we examined the ability of a bicistronic DNA vaccine encoding two malarial erythrocytic-stage candidate antigens, apical membrane antigen 1 (AMA-1) and mero-

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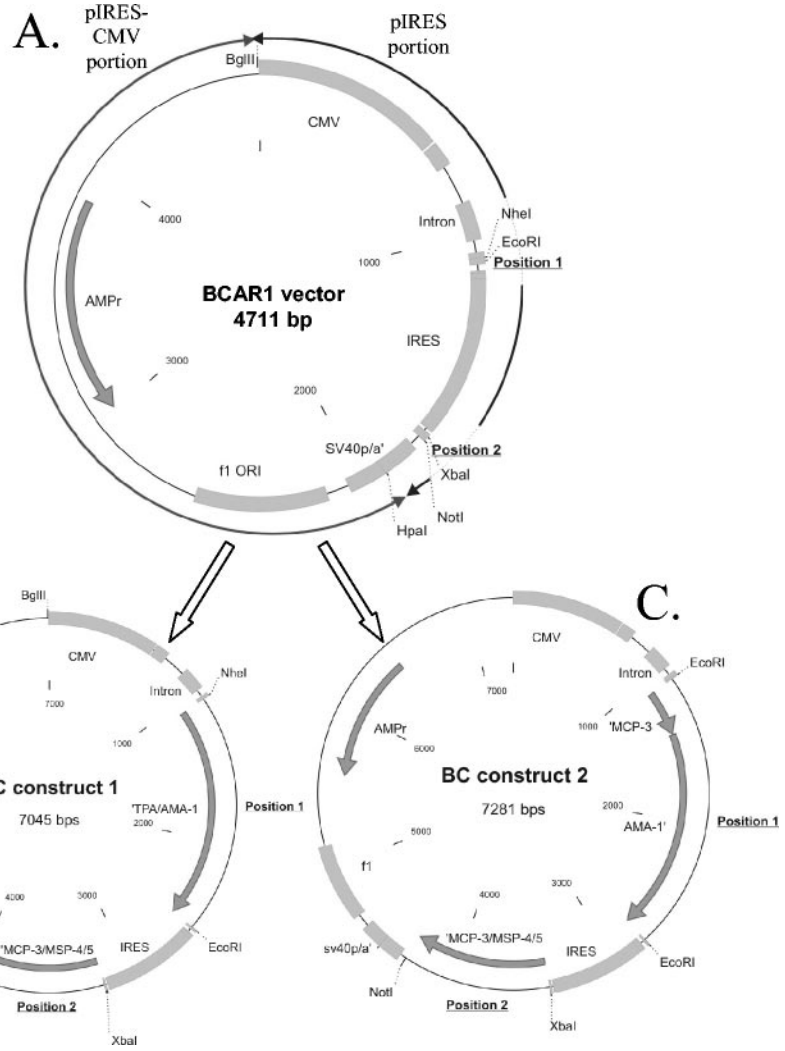


FIG. 1. (A) The prepared BCAR1 vector used for inserting AMA-1 and MCP-3–MSP4/5. The source of vector DNA used for the final BCAR1 vector is indicated by lines on the outer edges of the vector maps. The left portion was obtained from the pIRES-CMV vector, while the right portion was obtained from the pIRES vector (Clontech). The sequences inserted into the BCAR1 vector to produce BC construct 1 and BC construct 2 were amplified by PCR from previously constructed VR1020–AMA-1 and VR1012–MCP-3–MSP4/5 expression vectors. (B) BC construct 1 contained a TPA secretion signal fused to AMA-1 in position 1, followed by an IRES sequence, and a secretory MCP-3 chemokine sequence fused to MSP4/5 in position 2 of the vector. (C) BC construct 2 contained a secretory MCP-3 chemokine sequence fused to AMA-1 in position 1 and an IRES sequence, followed by the MCP-3–MSP4/5 sequence in position 2 of the vector. SV40, simian virus 40.

zoite surface protein 4/5 (MSP4/5), to express both antigens in vitro and in vivo and to induce antibody and splenic T-cell responses after immunization of mice. The effect of bicistronic immunization of mice on parasitemia after lethal erythrocytic-stage challenge was also assessed.

MATERIALS AND METHODS

Creation of bicistronic plasmids. (i) Bicistronic vector preparation. A bicistronic pIRES vector backbone was obtained from Clontech (Palo Alto, Calif.). The pIRES vector was first digested with HpaI and BglII restriction enzymes to remove the neomycin resistance gene cassette contained within this vector. This resulted in a 1,920-bp fragment with two multiple cloning sites and an IRES sequence. The pIRES-CMV vector was kindly provided by Stephen Hobbs (Institute of Cancer Research, London, United Kingdom) (13). This vector was also digested with HpaI and BglII to produce a 2,791-bp fragment containing an ampicillin resistance gene and a portion of the simian virus 40 polyadenylation sequence. This fragment was ligated to the 1,920-bp fragment to produce a 4,711-bp empty bicistronic vector (BCAR1 vector) (Fig. 1A).

(ii) BC construct 1 construction. Bicistronic construct (BC construct) 1 contained the DNA encoding the AMA-1 (Ser 22-to-Gln 479) ectodomain of *Plasmodium chabaudi adami* DS fused to a tissue plasminogen activator (TPA) secretion signal in the first position of the BCAR1 vector and the DNA encoding MSP4/5 (Met 1 to Ser 190) of *P. c. adami* DS fused to the monocyte chemotactic protein 3 (MCP-3) DNA coding sequence in the second position of the vector (Fig. 1B).

The 892-bp MCP-3–MSP4/5 sequence was amplified by PCR from the construct generated as described in reference 27, with use of the oligonucleotides GAAGTTCTAGAATGAGGATCTCTGCCACG (containing an XbaI site) and GAAGTGCGGCCGCTTATGAATCTGCACTGAG (containing a NotI site). The resulting PCR product was digested with XbaI and NotI enzymes and ligated into position 2 of the BCAR1 vector.

The AMA-1 ectodomain was amplified from *P. c. adami* DS genomic DNA by PCR with use of oligonucleotides GGGAAGATCTTCCGAAGGTACAGATA and GAAGTAGATCTTTACTGATTTATGGACT. The resultant AMA-1 fragment was then digested with BglII and inserted into the BglII site of VR1020 (VICAL, San Diego, Calif.) in frame with the TPA secretion signal. The TPA–AMA-1 sequence was then amplified by PCR from the VR1020–AMA-1 construct with use of oligonucleotides CGCGGAGCTAGCATGGATGCAAT

GAAGAGA and GAAGTGAATTCTTACTGATTTATTGGACT, with the resulting product being digested with NheI and EcoRI. The BCAR1 vector (containing the MCP-3–MSP4/5 sequence) was digested with NheI and EcoRI, and the TPA–AMA-1 sequence was inserted to complete the construct, which was designated BC construct 1 (Fig. 1B). The BCAR1 vector (Fig. 1A) was also produced with TPA–AMA-1 in position 1 alone, or with MCP-3–MSP4/5 in position 2 alone, with use of the same restriction enzyme positions described above. Both these single-antigen constructs (within the BCAR1 vector) were used in control experiments.

(iii) BC construct 2 construction. BC construct 2 contained the AMA-1 ectodomain sequence fused to the MCP-3 sequence in the first position of the BCAR1 vector and MSP4/5 fused to the MCP-3 sequence in the second position of the vector. BC construct 1, lacking the TPA–AMA-1 sequence in the first multiple cloning position but containing MCP-3–MSP4/5 in position 2, was used as a backbone for BC construct 2 (Fig. 1C).

The AMA-1 ectodomain was amplified by PCR from the VR1020–AMA-1 construct (as described for the construction of BC construct 1) with use of oligonucleotides ATGATGGGATCCGAAGGTACAGATAAT and GAACTGGATCCTTACTGATTTATTGGACT, and the product was digested with BamHI. The PCR product was then inserted into the BamHI site of the VR1012–MCP-3 vector (27) to produce a VR1012–MCP-3–AMA-1 construct. The MCP-3–AMA-1 (1,695-bp) sequence was amplified using oligonucleotides GAAGTGAATTCATGAGGATCTCTGCCACG and GAAGTGAATTCTTACTGATTTATTGGACT and digested with EcoRI. The BC construct 1 backbone was digested with EcoRI, and the MCP-3–AMA-1 DNA sequence was inserted into the first multiple cloning site to produce BC construct 2 (Fig. 1C).

Expression and purification of recombinant proteins. (i) Purification of MSP4/5. Expression and purification of the recombinant MSP4/5 ectodomain protein were performed as described in reference 2. Briefly, the pTrcHis-A/MSP4/5 vector was transfected into *Escherichia coli* BL21(DE3) (Novagen, Milwaukee, Wis.) for expression of recombinant MSP4/5 protein. Large-scale purification of the recombinant protein was performed using Talon metal affinity resin (Clontech) according to the manufacturer's instructions.

(ii) Purification of AMA-1. The *E. coli* strain JPA101, containing the AMA-1 ectodomain sequence in the expression vector pDS56/RBSii, was kindly provided by Robin Anders (La Trobe University, Melbourne, Australia) (1). A colony of *E. coli* containing the plasmid encoding the AMA-1 ectodomain was used to inoculate 50 ml of Superbroth (3.5% tryptone, 2% yeast extract, 0.5% NaCl) with 50 μg of ampicillin/ml. The 50-ml culture was grown overnight at 37°C, used to inoculate 500 ml of Superbroth (with 100 μg of ampicillin/ml), and incubated for a further 2 h. Induction of AMA-1 protein expression was performed by addition of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Progen, Darra, Australia), and incubation continued for 3 h at 37°C. The culture pellet was collected by centrifugation for 10 min at 3,000 × *g* at 4°C.

The purification of the AMA-1 ectodomain was performed under denaturing conditions. The culture pellet was resuspended in 20 ml of extraction buffer, pH 8 (50 mM $NaH_2PO_4 \cdot 2H_2O$, 6 M guanidine-HCl [pH 8], 300 mM NaCl) with 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Mo.). The resuspended pellet was then applied to a French press for three cycles at a pressure of 4 tons. The lysate was then centrifuged at 11,000 × *g* for 15 min at 4°C, and the pH was corrected to 8 by addition of 5 M NaOH. The lysate was then applied to 2 ml of Talon metal affinity resin (Clontech) and incubated with rotation for 1 h at 4°C. The resin was then washed three times with 50 ml of extraction buffer, pH 8, before being applied to a column. The final wash step involved the addition of 5 mM imidazole to the extraction buffer before elution. The AMA-1 was then eluted from the resin with use of 1× elution buffer, pH 7 (45 mM $NaH_2PO_4O \cdot 2H_2O$, 5 M guanidine-HCl, 270 mM NaCl, 150 mM imidazole).

To refold the AMA-1 ectodomain, the eluted protein was dialyzed at 4°C. The dialysis buffer (20 mM Tris-HCl, pH 8) was changed three times over 48 h to remove any remaining elution buffer. The dialysis tube containing the AMA-1 was then immersed in refolding buffer (1 mM reduced glutathione, 0.2 mM oxidized glutathione, 20 mM Tris-HCl, pH 8) in a volume 25 times greater than the AMA-1 solution in the dialysis tube. This mixture was then degassed by vacuum, sealed under nitrogen, and dialyzed overnight at 4°C. The refolded AMA-1 protein was then stored at −80°C in 50% glycerol.

Mammalian cell transfection with bicistronic DNA plasmids. Bicistronic plasmid constructs were tested for expression in COS 7 cells prior to use in mice. Freshly grown COS 7 cells were seeded at 2×10^5 cells per 35-mm-diameter tissue culture well. Cells were grown in complete RPMI 1640 (Invitrogen, Carlsbad, Calif.) containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. COS 7 cells were then incubated in 5% CO_2 until 80% confluent. Three micrograms of plasmid DNA was used to transfect COS 7 cells with use of Lipofectamine (Invitrogen) in serum-free RPMI 1640 according to the manufacturer's instructions. Serum-free RPMI 1640 was changed to complete RPMI 1640 24 h after transfection. After incubation for a further 2 days at 37°C the cells were washed with phosphate-buffered saline (PBS), medium was replaced with serum-free RPMI 1640 (to remove any FCS that may have masked protein detection by subsequent Western blot analysis), and cells were grown for a further 24 h. The supernatant was then collected and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

SDS-PAGE and Western blotting. Protein and COS 7 supernatants were fractionated by SDS-PAGE on 12% (vol/vol) polyacrylamide gels under reducing conditions and transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked in 5% milk powder in PBS and 0.05% Tween 20 (Sigma) (PBS-T) overnight at 4°C. The membranes were probed using an anti-MSP4/5 rabbit antibody or anti-AMA-1 rabbit antibody, followed by an anti-rabbit immunoglobulin (Ig) conjugated to horseradish peroxidase (Silenus Laboratories, Melbourne, Australia). The reactive antibodies were then visualized by enhanced chemiluminescence (Amersham, Piscataway, N.J.).

ELISA. Antibody reactivity after vaccination was tested with recombinant MSP4/5 or refolded AMA-1 protein and measured by enzyme-linked immunosorbent assay (ELISA). Nunc Maxisorp (Nunc, Roskilde, Denmark) ELISA plates were coated with 0.1 ml of recombinant MSP4/5 or AMA-1 (1 μg/ml)/well overnight at 4°C with carbonate-bicarbonate buffer, pH 9.6. Plates were washed with PBS-T, followed by blocking overnight at 4°C in 5% skim milk powder and PBS-T. Plates were again washed, and diluted sera were incubated at 37°C for 2 h. After plates were washed again with PBS-T, total humoral responses were obtained with horseradish peroxidase-conjugated sheep anti-mouse Ig (Silenus) diluted 1:2,000 and incubated for 1 h, followed by washing and addition of substrate. After a final washing, the ELISA product was developed by addition of the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma). Absorbance was measured at 450 nm, and titers were defined as the highest dilution required for an absorbance of 0.2.

Isolation of plasmid DNA and construction of vaccination cartridges. DNA plasmid constructs were transfected into *E. coli* DH5α and grown on solid agar medium containing 50 μg of ampicillin/ml prior to inoculation into liquid Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 50 μg of ampicillin/ml. Inoculated LB medium (with 50 μg of ampicillin/ml) was grown with shaking at 37°C overnight. Plasmid preparation and endotoxin removal were performed using the Qiagen endotoxin-free plasmid Giga kit according to the manufacturer's instructions (Qiagen, Inc., Valencia, Calif.). Purified DNA was precipitated onto gold microcarriers, and these were attached to plastic supports per the manufacturer's recommendations (Bio-Rad Laboratories, Hercules, Calif.). DNA was combined with gold microcarriers at a ratio of 100 μg of DNA to 50 mg of carriers. Each projectile contained approximately 1 μg of DNA.

Mice and vaccination. All mice were BALB/c, female, and 5 to 6 weeks of age at the time of first vaccination. DNA-vaccinated mice received three immunizations at 2-week intervals. For intraepidermal (i.d.) DNA vaccination, the abdominal region was shaved and particles containing 1 μg of DNA were delivered by the Helios gene gun (Bio-Rad Laboratories) with a pulse of helium gas at 400 lb/in^2. Intramuscular (i.m.) DNA plasmids were delivered into the tibialis anterior muscle (100 μg total) in PBS.

In vitro spleen cell proliferation. Spleen cell proliferation was performed as described in reference 26. Briefly, recombinant AMA-1 and MSP4/5 were further purified using Detoxi-Gel (Pierce, Rockford, Ill.) to remove any endotoxin contamination and reduce nonspecific proliferation according to the manufacturer's instructions. The recombinant proteins were used to stimulate splenocytes at a final concentration of 5 μg/ml. As a control for cell viability, splenocytes were stimulated with concanavalin A (Sigma) at a final concentration of 2.5 μg/ml. Splenocytes were cultured for 96 h in flat-bottomed microtiter plates in triplicate at a final concentration of 5×10^6 cells/ml (10^6 cells/well) and pulsed with 1 μCi of [^{3}H]thymidine (Amersham Biosciences Corp.)/well for 18 h before harvesting. The splenocytes were harvested onto glass fiber filter mats (Saktron Instruments Inc., Sterling, Va.) with use of an automated cell harvester (Saktron), and incorporated radioactivity was measured using a liquid scintillation counter (Perkin-Elmer Life Sciences, Wellesley, Mass.).

Phagocytosis assays. Phagocytosis assays were performed according to the method described in reference 25 with the following modifications. Macrophages were obtained from BALB/c mice by peritoneal lavage with 9 ml of ice-cold 0.34 M sucrose in PBS (pH 7.2). The cells were then centrifuged at 1,000 × *g* for 10 min at 4°C. Peritoneal cell exudates were resuspended in complete RPMI 1640 (Invitrogen) supplemented with 10% FCS, 2 mM glutamine (Invitrogen), 0.05 mM 2-mercaptoethanol (Sigma), and penicillin-streptomycin (100 U/ml; Invitrogen) to a final concentration of 2×10^6 cells/ml. Eight-well chamber slides

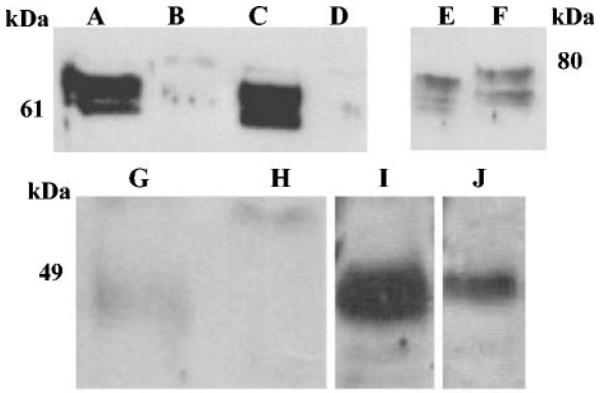
(Nalge Nunc International Corp., Naperville, Ill.) were used, with 4×10^5 macrophages added to each well. The macrophages were allowed to adhere for 2 h at 37°C with 5% CO_2. After 2 h, nonadherent T cells were removed by careful washing with 1 ml of 37°C RPMI 1640. Fresh complete RPMI 1640 was added, and macrophages were left to adhere for a further 2 h. During this time, fresh *P. c. adami* DS-infected red blood cells (IRBC; 10^8 IRBC/ml, containing trophozoites and schizonts at approximately 40 to 50% parasitemia) in complete RPMI 1640 were purified on Ficoll gradients (Amersham). The IRBC were washed twice with complete RPMI 1640. After washing, IRBC pellets were placed into 1.5-ml centrifuge tubes in 15-μl aliquots. Thirty microliters of PBS and 1 μl of sera obtained from groups vaccinated with the bicistronic constructs and empty vector controls were then added to the IRBC pellets and incubated for 1.5 h at 37°C with shaking. After 1.5 h, IRBC and sera were added to adherent macrophages and incubated for 3 h at 37°C with 5% CO_2. The eight-well slides were then washed four times with PBS to remove nonadherent macrophages and noningested IRBC. Noningested (but adherent) IRBC were lysed by incubation of the slides with cold water for 20 s, followed by washing with PBS. The eight-well slides were then fixed and stained using Kwik Diff staining solution (Terno Shandon, Pittsburgh, Pa.). The percentage of macrophages ingesting IRBC was quantified by examination of 300 cells per individual sample by light microscopy.

Infection of mice, blood sampling, and parasitemia measurements. Blood from an infected mouse with a known parasitemia (1 to 10%) was taken and immediately diluted in PBS to give the required dosage (10^5 infected RBC/dose). Mice were infected by intraperitoneal injection at day 0, and parasitemia was assessed from day 6 through the period of crisis until the resolution of parasitemia. Infection levels were assessed by Giemsa staining of tail smears. Mean peak parasitemia levels and days to peak parasitemia were compared with use of a Student *t* test.

Analysis of survival curves. Survival curves for vaccinated and control mice were compared using the Mantel-Haenszel test. Statistical analysis was performed using Prism 3.02 software (GraphPad, San Diego, Calif.).

RESULTS

Expression of protein encoded by bicistronic plasmids in vitro. The ability of mammalian cells to secrete MSP4/5 or AMA-1 after transfection with bicistronic DNA vaccine plasmids was tested in COS 7 cells. Proteins encoded by the constructs were secreted into the culture supernatant in vitro (Fig. 2) and detected by Western blotting after probing with either anti-AMA-1 or anti-MSP4/5 rabbit sera. BC construct 1 contained the TPA–AMA-1 sequence in the first position, followed by MCP-3–MSP4/5 in the second position after the IRES sequence. AMA-1 protein secretion from COS 7 cells transfected with BC construct 1 was detected (Fig. 2C), as was AMA-1 protein from COS 7 cells transfected with the VR1020–AMA-1 plasmid as a positive control (with AMA-1 secreted by the TPA signal sequence contained in both vectors [Fig. 2A]). The MCP-3–AMA-1 fusion protein encoded in the first position of BC construct 2 was also secreted into the cell supernatant after transfection into COS 7 cells and detected via Western blotting (Fig. 2E). A VR1012–MCP-3–AMA-1 construct was also included as a positive control and detected in the COS 7 supernatant via Western blotting (Fig. 2F). The secretion of the MCP-3–MSP4/5 fusion protein (from the MSP4/5 sequence contained in the second cloning position after the IRES sequence) was also detected after transfection of COS 7 cells with both BC construct 1 and BC construct 2. However, the signal strength of the secreted MCP-3–MSP4/5 fusion protein was significantly lower with BC construct 1 (containing a TPA leader sequence in position 1 [Fig. 2G]) than with BC construct 2 (with an MCP-3 leader sequence in position 1 [Fig. 2J]) and the VR1012–MCP-3–MSP4/5 construct expressing only MCP-3–MSP4/5 [Fig. 2I]). Control supernatants collected from COS 7 cells after transfection with bicistronic DNA vaccine vectors alone did not react with specific rabbit sera to each recombinant protein (Fig. 2B, D, and H).



A VR1020/AMA-1 control
B VR1020 vector
C TPA/AMA-1 in Position 1 of BC construct **1**
D BCAR1 vector
E MCP-3/AMA-1 in Position 1 of BC construct **2**
F MCP-3/AMA-1 control
G MCP-3/MSP4/5 in Position 2 of BC construct **1**
H BCAR1 vector
I VR1012/MCP-3/MSP4/5 control
J MCP-3/MSP4/5 in Position 2 of BC construct **2**

FIG. 2. Western blot of supernatants taken from COS 7 cells transfected with bicistronic and monocistronic DNA vectors containing antigen-encoding inserts. Western blot analyses were carried out using equivalent proportional volumes of supernatant loaded per lane. The Western blot was probed with anti-AMA-1 rabbit sera (A to F) or anti-MSP4/5 rabbit sera (G to J). Proteins expressed by cells transfected with each construct were secreted into the culture supernatant. Control vectors (B, D, and H) not containing inserts did not react with rabbit sera. The sizes of the expressed AMA-1 (80-kDa) and MCP-3–MSP4/5 (49-kDa) proteins are indicated.

Humoral responses in mice vaccinated with bicistronic DNA vaccine constructs. The bicistronic constructs were used to vaccinate mice by either i.m. injection or i.d. injection with the gene gun. The resulting antibody responses were measured 2 weeks after the third vaccination (week 6) by ELISA with use of recombinant MSP4/5 protein or recombinant refolded AMA-1 protein. As shown in Fig. 3, mice vaccinated with BC construct 1 via i.d. (Fig. 3A) or i.m. (Fig. 3C) routes produced a detectable IgG response to both AMA-1 and MSP4/5 by ELISA, with the titer to AMA-1 (untargeted) being significantly higher than the titer observed with MSP4/5 by the i.d. route (Fig. 3A; $P = 0.02$). A higher mean IgG antibody titer to AMA-1 than to MSP4/5 was also detected after vaccination with BC construct 1 via the i.m. route, although this was not statistically significant (Fig. 3C; $P = 0.15$).

Vaccination of mice by the i.d. route with MCP-3–AMA-1 in position 1 of BC construct 2 did not promote an enhanced antibody response to AMA-1, whereas the mean MSP4/5 antibody response was greater (Fig. 3B). The antibody response to recombinant AMA-1 was low (titer, 1/200) and unable to be definitively distinguished above the background level of reactivity observed with negative control serum (titer, 1/100). This is in contrast to the use of a TPA–AMA-1 sequence in the first position of BC construct 1, which resulted in high antibody

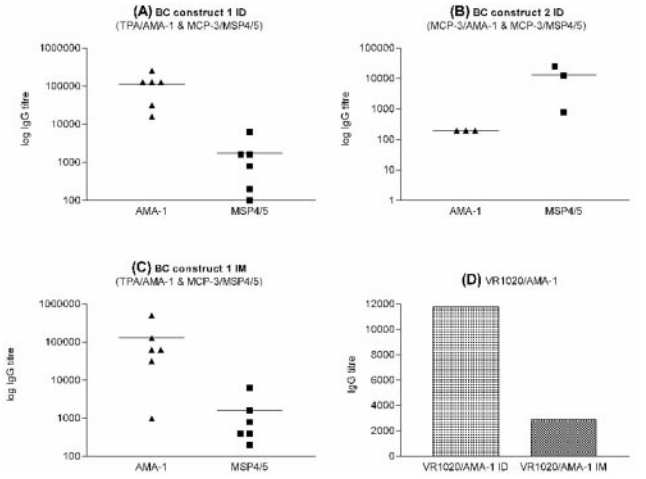


FIG. 3. IgG responses of mice vaccinated with bicistronic and monocistronic DNA vaccine constructs. IgG antibody responses were measured by ELISA. Vaccines were delivered using either 1 μg of DNA i.d. (gene gun) or 100 μg of DNA i.m. Graphs A, B, and C show data from sera taken from individual mice reacting to both MSP4/5 and AMA-1 recombinant protein after vaccination with a single bicistronic construct. The mean titer is indicated by a bar. (D) Pooled sera from five mice vaccinated with VR1020–AMA-1 via both i.d. and i.m. routes. Mice vaccinated with vectors not containing AMA-1 or MSP4/5 inserts did not mount an antibody response (data not shown).

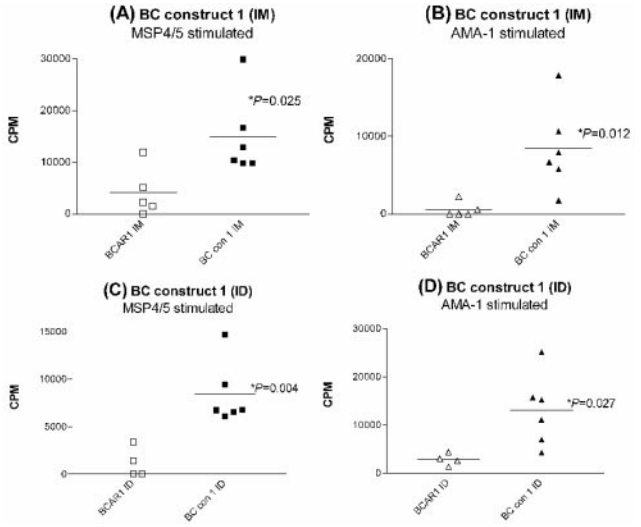


FIG. 4. Proliferation of splenocytes primed with bicistronic vectors. Shown is in vitro proliferation of splenocytes from individual BALB/c mice vaccinated i.d. with the gene gun or i.m. by injection. Mice were vaccinated with BC construct 1 (BC con 1) or the empty BCAR1 vector three times at 2-week intervals. Splenocytes were harvested 10 days after the final vaccination. BC construct 1-primed splenocytes were stimulated with AMA-1 or MSP4/5 and harvested at 72 h, after [^{3}H]thymidine was added 18 h previously. [^{3}H]thymidine incorporated by cells was then measured. Splenocytes from all individual cultures responded to concanavalin A stimulation (data not shown). The mean counts per minute (bars) are shown. Statistical analysis was performed using an unpaired *t* test.

responses to AMA-1 but low responses to MSP4/5 antibodies when delivered via i.d. or i.m. routes (Fig. 3A and C). Therefore, BC construct 2 was excluded from future experiments due to the lack of a detectable antibody response to AMA-1.

As a comparative control experiment, mice (five per group) were vaccinated with the untargeted monocistronic VR1020–AMA-1 construct via i.d. (gene gun) or i.m. routes. Control mice (five per group) vaccinated with the VR1020 vector via i.m. and i.d. routes showed no antibody response to AMA-1 (data not shown). For this control experiment, sera were pooled and an ELISA was performed in response to refolded recombinant AMA-1 (Fig. 3D). Although these data cannot be directly compared statistically to the data generated by the bicistronic constructs containing AMA-1 (Fig. 3A and C), the results suggest that high antibody production to AMA-1 can be induced by using a bicistronic construct relative to the VR1020–AMA-1 monocistronic construct (Fig. 3D), i.e., bicistronic expression from position 1 of the construct was not deleterious to antigenicity. Similar high titers can be induced by vaccination of mice with a monocistronic MCP-3–MSP4/5 vaccine (27). Vaccination using the empty DNA vaccine BCAR1 control vector did not produce a detectable IgG response to MSP4/5 or AMA-1 protein (data not shown).

Cellular immune responses induced by bicistronic DNA vaccination. To evaluate cellular responses to *P. c. adami* DS antigens induced by bicistronic vaccination, groups of mice were vaccinated i.d. or i.m. with BC construct 1 (containing TPA–AMA-1 and MCP-3–MSP4/5) or empty BCAR1 DNA control vectors. Ten days after the final vaccination, cell proliferation assays were performed on splenocytes from individual mice stimulated with recombinant AMA-1 and MSP4/5 protein. Significant levels of proliferation were observed after splenocytes were stimulated with MSP4/5, previously primed via the i.m. (Fig. 4A; $P = 0.025$) and i.d. (Fig. 4C; $P = 0.004$) routes, relative to the proliferation observed with splenocytes from mice primed with empty BCAR1 vector control DNA. Stimulation of splenocytes with AMA-1 also resulted in a significant increase in proliferation after vaccination by both the i.m. (Fig. 4B; $P = 0.012$) and i.d. (Fig. 4D; $P = 0.027$) routes over that observed with cells from mice vaccinated with empty vector DNA. No significant differences in the level of proliferation were observed between the routes of vaccination.

We were interested to determine whether T-cell responses were influenced by monocistronic delivery relative to bicistronic vaccination. Figure 5 shows a comparison of proliferation of splenocytes taken from mice vaccinated i.m. with either the BCAR1 empty vector, monocistronic TPA–AMA-1 (in position 1 of BCAR1), monocistronic MCP-3–MSP4/5 (in position 2 of BCAR1), or BC construct 1. Vaccination with BC construct 1 resulted in significantly greater splenocyte proliferation in response to specific antigen compared to that for both monocistronic constructs and the empty vector control. With MSP4/5 stimulation, splenocyte proliferation by cells from mice given BC construct 1 was significantly higher than that observed with cells from mice vaccinated with monocistronic MCP-3–MSP4/5 ($P = 0.01$). With AMA-1 stimulation, the level of splenocyte proliferation after vaccination with BC construct 1, relative to vaccination with the monocistronic TPA–AMA-1, was enhanced, although this was not statistically significant ($P = 0.06$). These results suggest that codelivery of AMA-1 and MSP4/5 in BC construct 1 leads to a synergistic T-cell response to both antigens.

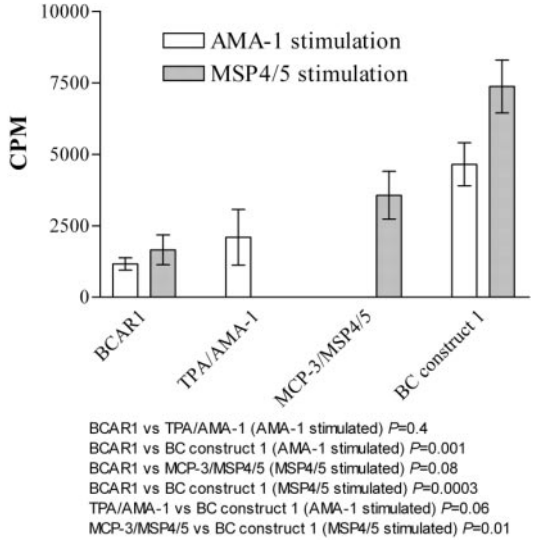


FIG. 5. Proliferation of splenocytes primed with bicistronic vectors via the i.m. route. Shown is in vitro proliferation of splenocytes from individual BALB/c mice (six per group) vaccinated i.m. by injection three times at 2-week intervals with either BC construct 1, BCAR1, monocistronic TPA–AMA-1, or monocistronic MCP-3–MSP4/5 (in the BCAR1 vector). Splenocytes were harvested 10 days after the final vaccination. BC construct 1-primed splenocytes were stimulated with AMA-1 or MSP4/5 and harvested at 72 h, after [^{3}H]thymidine was added 18 h previously. [^{3}H]thymidine incorporated by cells was then measured. Splenocytes from all individual cultures responded to concanavalin A stimulation (data not shown). The standard errors of mean counts per minute (stimulated minus unstimulated background counts per minute) are shown. Statistical analysis was performed using an unpaired *t* test.

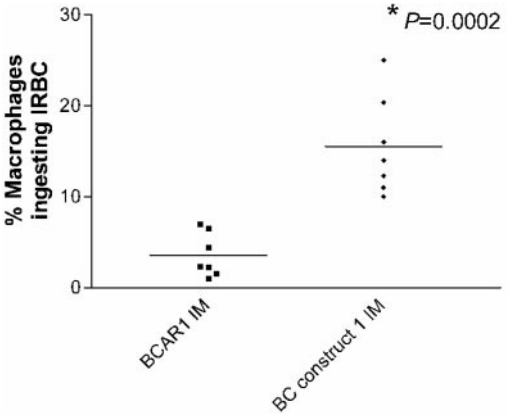


FIG. 6. Phagocytosis of *P. c. adami*-IRBC preincubated with sera from individual mice vaccinated i.m. with BCAR1 or BC construct 1. The percentage of macrophages containing IRBC was calculated from a total of 300 macrophages counted for each mouse sample. The means (solid bars) are shown. Groups were compared using a paired *t* test. *, significantly different from i.m. BCAR1 control vector.

Phagocytosis of *P. c. adami* DS-IRBC by macrophages after incubation with sera from mice vaccinated i.m. with BC construct 1. Opsonization of IRBC and subsequent internalization and destruction by macrophages have been shown to be a major factor contributing to a reduction in parasitemia during crisis in *P. chabaudi* mouse models (24–26). The opsonizing capacity of prechallenge sera taken from mice vaccinated i.m. with BC construct 1 or the empty BCAR1 vector was assessed. Figure 6 shows that the macrophages incubated with sera from mice vaccinated i.m. with BC construct 1 ingested significantly more IRBC than did macrophages incubated with sera from mice vaccinated with the empty BCAR1 ($P < 0.001$) vector. Sera from mice vaccinated i.d. with BC construct 1 also opsonized IRBC (data not shown).

Efficacy of the bicistronic DNA vaccine. (i) Trial 1. Trial 1 contained six female BALB/c mice per group vaccinated with BC construct 1. Mice were vaccinated i.m. (100 μg) by injection or i.d. (1 μg) with the gene gun three times at 2-week intervals and challenged with 100,000 *P. c. adami* DS-IRBC 2 weeks after the final vaccination. This trial represents an extremely stringent test of a vaccine due to the virulent nature of the challenge with *P. c. adami* DS. Figure 7 shows survival curves of mice challenged with lethal *P. c. adami*. There were no significant differences in survival between mice vaccinated i.m. or i.d. with the BC construct 1 and control mice, with all mice dying by day 13 (Fig. 7A and B). However, 33% of control mice died at day 10, compared to the first deaths occurring at day 11 for mice vaccinated with the BC construct 1 i.m. (Fig. 7A). The parasitemia levels of mice challenged in this trial, however, were influenced by the i.m. administration of bicistronic vectors, regardless of the lack of survival. Figure 7A.i shows the percent parasitemia measured from day 6 postinfection in i.m. vaccinated mice. No significant differences were found in the peak parasitemia levels between mice vaccinated with BC construct 1 i.m. and the control vaccinated with BCAR1 i.m. at day 9 postinfection (Fig. 7A.i). However, four out of six control mice reached their peak parasitemia at day 9, with the parasitemia of two remaining mice continuing to rise

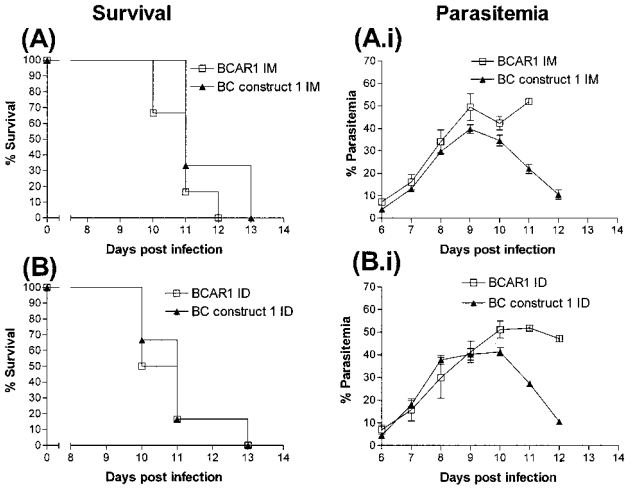


FIG. 7. (A and B) Bicistronic trial 1 survival curves. Six mice per group were vaccinated i.m. by injection with 100 μg of DNA or 1 μg via gene gun three times at 2-week intervals. Mice were challenged with 100,000 *P. c. adami* DS-IRBC. There were no significant differences between survival curves of control mice and those of vaccinates, as determined by the areas under the curves with use of the Mantel-Haenszel test for comparing survival curves. (A.i and B.i) Bicistronic trial 1 parasitemia curves. Smears were taken from individual mice (six mice per group) from day 6 postinfection, with 300 to 400 cells counted per smear.

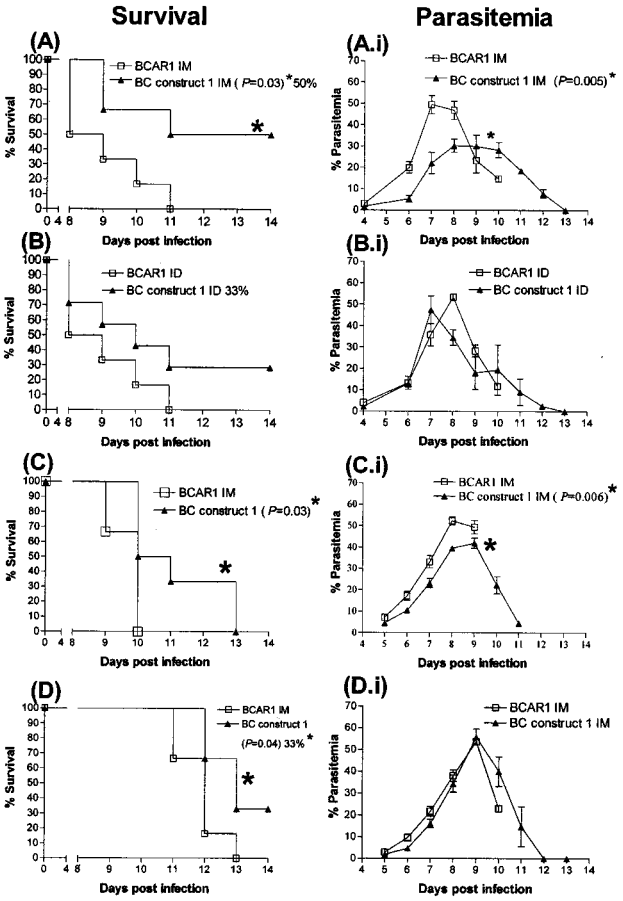


FIG. 8. (A to D) Bicistronic trial 2 to 4 survival curves. Six mice per group were vaccinated i.m. by injection with 100 μg of DNA or 1 μg via gene gun three times at 2-week intervals. Mice were challenged with 100,000 *P. c. adami* DS-IRBC. Significant differences between survival curves of control mice and those of vaccinates were determined by the areas under the curves with use of the Mantel-Haenszel test for comparing survival curves. The percent survival is shown in panels A to D; there were no survivors in panel C. (A.i to C.i) Bicistronic trial 2 to 4 parasitemia curves. Smears were taken from individual mice (six mice per group) from day 4 postinfection, with 300 to 400 cells counted per smear. There were no significant differences in peak parasitemia for survival curve D (data not shown). Significant differences in survival and peak parasitemias between controls and vaccinates are indicated by an asterisk (unpaired *t* test).

until day 11 (data not shown). In contrast, there was a sharp drop in parasitemia by day 12 for the two surviving mice vaccinated with BC construct 1 i.m., which outlived BCAR1 i.m. vaccination control mice and had almost resolved parasitemia before death at day 13 (Fig. 7A.i). Vaccination with BC construct 1 i.d. did not result in a significant reduction in peak parasitemia compared to that for BCAR1 vector control animals (Fig. 7B.i).

(ii) Trials 2 to 4. Figure 8A and B show the survival (and parasitemia) curves from the second bicistronic vaccine trial. BCAR1 control mice in trial 2 followed a pattern of survival similar to that in trial 1, and all mice died by day 11 postchallenge regardless of vaccination route. Mice vaccinated with BC construct 1 i.m. showed a significantly enhanced survival compared to that of the control group vaccinated with BCAR1, with 50% of mice surviving (Fig. 8A; $P = 0.03$). There was no significant difference in kinetics of survival of mice between delivery of BC construct 1 via the i.d. route and the empty vector (Fig. 8B), although 33% of vaccinated mice survived. The survival found after BC construct 1 i.d. delivery in this experiment is comparable to that seen using a VR1012–MCP-3–MSP4/5 construct (expressing the same sequence as contained in position 2 of BC construct 1) delivered i.d. as a monocistronic construct (27). Vaccination via the i.d. route with VR1020–AMA-1 (containing the TPA–AMA-1 sequence as used in BC construct 1) has no effect on survival (data not shown).

The peak parasitemia of mice vaccinated i.m. with BC construct 1 was significantly reduced by an average of 19.4% compared to that of mice vaccinated i.m. with the empty control vector (Fig. 8A.i; $P = 0.005$). This does not occur when the antigens are delivered as monocistronic constructs with use of either VR1020–AMA-1 (data not shown) or MCP-3–MSP4/5 (27). As in trial 1, vaccination via the i.d. route did not have any effect in reducing the parasitemia, with the BC construct 1 group peaking before the empty vector control group (Fig. 8B.i). In i.d. vaccination with VR1012–MCP-3–MSP4/5 with use of the *P. c. adami* DS model, there is no effect on parasitemia, although survival is increased relative to that of empty vector control groups as reported previously (27).

The experiment involving the i.m. route of vaccination with the BC construct 1 vaccine was repeated a further two times to confirm the observations from trials 1 and 2 (Fig. 8C and D). In trial 3, a significant difference in the rate of survival was observed in mice vaccinated with BC construct 1 (Fig, 8C: $P = 0.03$) and the peak parasitemia of vaccinated mice was also significantly reduced by an average of 10.4% (compared to i.m. delivery of the empty control vector) (Fig. 8C.i; $P = 0.006$). In the fourth trial, as shown in Fig. 8D and D.i, the BC construct 1 vaccine had a significant effect on survival rate (33% surviving); however, there was no significant effect on parasitemia (Fig. 8D.i).

The data from these experiments suggest that codelivery of AMA-1 and MCP-3–MSP4/5 (with both antigens being secreted) in a bicistronic DNA vaccine construct by the i.m. route appears to significantly increase survival and reduce parasitemia.

DISCUSSION

In this study it has been shown that a bicistronic DNA vaccine vector can express two candidate malaria vaccine antigens, evoke humoral and cellular immune responses, reduce parasitemia, and partially protect mice against lethal *P. c. adami* DS challenge. The aim of this study was not to directly compare the efficacies of bicistronic and monocistronic vectors but to evaluate the potential of bicistronic vectors as a delivery platform for multivalent vaccines against malaria. To date, there are no reports evaluating the potential of bicistronic vectors to deliver malarial antigens, even though a multistage and multiantigen malarial vaccine is believed to be optimal to protect against malaria (8, 18).

The bicistronic constructs were shown to express both encoded antigens in vitro. The transfection of BC construct 1 (containing a TPA leader secretion signal in position 1 and the

MCP-3 sequence at position 2) into COS 7 cells resulted in the secretion of both the AMA-1 and MSP4/5 antigens in vitro. However, expression of MCP-3–MSP4/5 (position 2 of BC construct 1) by COS 7 cells was markedly reduced compared to that of AMA-1 in position 1 of this construct. This was supported by the observation that, after vaccination of mice with BC construct 1 (via i.d. and i.m. routes), low MSP4/5 antibody titers were detected, relative to an enhanced AMA-1 antibody response. Again, this emphasizes earlier observations that the dominant determinants of translation efficiency in bicistronic vectors are the arrangement and nature of coding sequences in the mRNA (12).

BC construct 2 was aimed at delivering two targeted malarial candidate antigens with use of the chemoattractant MCP-3, since we had found that an MCP-3 vector enhanced the survival of mice with use of MSP4/5 as a vaccine after DNA vaccination, relative to a VR1020 construct (27). It was hypothesized that the fusion of the MCP-3 sequence to both AMA-1 (position 1 of BC construct 2) and MSP4/5 (position 2 of BC construct 2) would recruit dendritic cells to the site of antigen expression and enhance priming of naïve T cells by both antigens (reviewed in reference 21). Transfection of BC construct 2 into COS 7 cells resulted in both antigens fused to MCP-3 being secreted into the supernatant, and this was detected via Western blotting. However, after vaccination of mice, a humoral response to MCP-3–AMA-1 could not be detected in vivo even though secretion was found to be possible in vitro. This does not exclude the possibility that a cellular response may have been evoked in the absence of antibody in vivo (reviewed in reference 7). It has been found that the nucleotide composition of genes contained within a bicistronic construct has a significant effect upon IRES-promoted translation (12). The results from the present study show that the design of the bicistronic vectors was critical to ensure efficient coexpression of encoded malaria antigens.

Vaccination with bicistronic vectors containing rodent malaria homologues of human candidate malarial antigens AMA-1, MSP4, and MSP5 induced immune responses to both antigens. The route of delivery (i.m. or i.d.) did not have any significant effects upon antibody titers or the level of T-cell proliferation when T cells were stimulated with AMA-1 or MSP4/5 antigens. These results show that the bicistronic malarial DNA vaccines primed the immune system with both antigens simultaneously, regardless of delivery route. B-cell-deficient mice can control erythrocytic-stage malaria infections by limiting parasite growth, emphasizing the importance of T-cell-mediated immunity in the mouse malaria model (30, 31). The generation of $CD4^+$ T-cell responses against *Plasmodium falciparum* erythrocytic infection is believed to be of primary importance, by acting as T helper cells for antibody responses as well as effector cells and by limiting parasite growth via antibody-independent cell-mediated immunity (reviewed in references 10 and 11).

Survival after i.m. bicistronic vaccination following challenge with lethal *P. c. adami* DS was variable and did not strictly correlate with significant reductions in parasitemia in challenge trials, although there was a trend toward a reduction in parasitemia compared to that for control animals over the four trials. Trials 2 to 4 in particular resulted in a significant delay in death in mice vaccinated with BC construct 1 i.m. In summary, over the six vaccine trials evaluating the BC construct 1, the survival rate in vaccinates was 7 of 36 mice, whereas 0 of 36 controls survived.

Several approaches could be used to optimize the bicistronic vaccination strategy in order to enhance survival and further reduce parasitemia. The gene sequences used in BC construct 1 (AMA-1 and MSP4/5) were the native *P. c. adami* DS gene sequences, which, like the *P. falciparum* genes, are highly A/T rich. Hoffman and Doolan (14) have optimized the codon usage of *P. falciparum* genes to more closely reflect codon usage in mammalian genes, resulting in a 5- to 40-fold enhancement of in vitro expression in mammalian cells and 5- to 100-fold-higher antibody titers in outbred mice (7, 14). This is one method of enhancement that could improve BC construct 1 efficacy. The use of a nonlethal strain of rodent malaria (such as *P. c. adami* DK) (20) would allow a single parameter, that of parasitemia, to be measured in the absence of the complications due to pathology associated with the *P. c. adami* DS challenge: this was found to be a key effect in the present study, and it appears that the *P. c. adami* DS model is not the optimal model to evaluate the effects of malaria vaccines on parasitemia. Reducing parasitemia is an important aim for a human malaria vaccine since this would reduce morbidity (and potential mortality) in human malaria (23).

The ability of bicistronic constructs in the present study to codeliver malarial antigens and induce immune responses in vivo after vaccination establishes a new approach to malaria vaccine design. The inclusion of MCP-3 in the second position of the bicistronic construct may have increased the presentation of the untargeted antigen AMA-1 within BC construct 1. The migration of dendritic cells (due to MCP-3 expression) to a single site of bicistronic antigen production may have contributed to vaccine efficacy (as both bicistronically expressed antigens were in the same cellular location), leading to enhanced immune responsiveness and significant reductions in parasitemia. The bicistronic delivery of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) in other systems has resulted in significant enhancement of responses to DNA vaccination. By use of a hepatitis C virus bicistronic DNA vaccine, it has been shown that the delivery of a bicistronic plasmid containing hepatitis virus antigens and GM-CSF can significantly enhance T-cell proliferation and antibody responses, compared to vaccination with two separate plasmids (4). This has also been shown for hepatitis B virus DNA vaccination (5). However, the codelivery of GM-CSF with 3,000 plasmids from the VR1020 *P. c. adami* DS genomic library, as separate monocistronic constructs, was not effective at reducing peak parasitemia after erythrocytic-stage challenge with *P. c. adami* DS (29). Monocistronic coadministration of *Plasmodium yoelii* circumsporozoite protein DNA plasmids and plasmids containing GM-CSF has been shown to result in increased $CD4^+$ and $CD8^+$ T cells, antibody production, and protection against sporozoite challenge in murine studies (33). The use of GM-CSF and $MSP\text{-}1_{42}$ as a DNA vaccine in rhesus monkeys resulted in a rapid induction of antibodies after the first dose but had no effect on the T-cell response (19). The delivery of cytokines such as GM-CSF, along with malarial candidate antigens or genomic-cDNA libraries in bicistronic vectors, may allow for a more efficient vaccine delivery system, as seen in hepatitis models. However, the efficiency of immune

responses to combinations of gene pairs within bicistronic constructs varies markedly between different constructs (12). Whether the enhancement of DNA vaccines by cytokines can be applied to bicistronic malarial erythrocytic-stage vaccines still remains to be tested.

Our results showing the induction of both antibody and T-cell responses against, as well as the reduction in parasitemia for, lethal *P. c. adami* DS challenge in mice demonstrate that the delivery of malarial antigens via bicistronic vectors is feasible in the murine model. The optimization and refinement of bicistronic DNA vaccines for malaria will now need to occur. This may include codon optimization, codelivery of cytokines, viral boosting, and testing in different murine malaria models.

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