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Mammalian cell expression of malaria merozoite surface proteins and experimental DNA and RNA immunisation

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Abstract

The gene for a 45 kDa merozoite surface protein (MSA-2) of the human malaria parasite *Plasmodium falciparum* was PCR amplified and cloned into eukaryotic expression vectors VR1012 and pcDNA3 to yield plasmids P1 and P2, respectively. The coding sequences for two N-terminal fragments of the 185 kDa merozoite surface protein (MSA-1) gene were similarly PCR amplified and cloned into vectors VR1020 and VR1012 to yield plasmids P3 and P4, respectively. The MSA-1 signal peptide sequence, present in P4, was replaced with the human tissue plasminogen activator signal sequence in P3. The four plasmids expressed the cloned genes under the control of the cytomegalovirus promoter and carried 3′ bovine growth hormone termination/poly A signals. P1, P3 and P4 also contained the cytomegalovirus intron A enhancer sequence. MSA-1 expression was more readily detected than MSA-2 in Cos cells transfected with P3/P4 and P1/P2 respectively. The MSA-2 gene was also cloned into the phagemid pBluescript IISK$^+$ with and without a 3′ poly A tail composed of 35 A residues. MSA-2 was synthesised in HeLa cells infected with a recombinant vaccinia virus carrying T7 RNA polymerase when MSA-2 recombinant pBluescript was transfected into the cells. Inoculation with P1 intramuscularly or intradermally and with P2 intradermally into rabbits led to the production of antibodies to MSA-2 detectable by immunofluorescence and Western blotting. Antibodies were also produced against MSA-1 after intramuscular/intradermal inoculation with P3 and P4. Inoculation of rabbits with MSA-2 mRNA yielded better antibody titres when a poly A tail was present. Antibody levels were maintained for >9 weeks after the final immunisation. However the immune sera failed to inhibit in vitro parasite growth.

Keywords: DNA vaccine; Eukaryotic gene expression; Malaria; Surface protein; RNA vaccine; (*Plasmodium falciparum*)

1. Introduction

The development of a malaria vaccine is hindered by: (1) the presence of several parasite forms with distinct target antigens that are susceptible to different immune effector mechanisms; (2) sophisticated parasite strategies for immune evasion [1]; and (3) the lack of effective vaccine delivery systems and ad-

Abbreviations: aa, amino acid; BGH, bovine growth hormone; CMV, cytomegalovirus; DOTAP, *N*-[1-(2,3-D-dioleoyloxy) propyl]-*N*,*N*,*N*-(trimethylammonium) methyl sulphate; EGF, epidermal growth factor; ER, endoplasmic reticulum; GPI, glycosylphosphatidyl inositol; HBS, HEPES buffered saline; IFA, immunofluorescence assay; mab, monoclonal antibody; MSA, merozoite surface antigen; RBC, red blood cell; tPA, tissue plasminogen activator

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juvants. The demonstration that plasmids containing foreign genes under the control of a mammalian cell or virus promoter can directly transfect mammalian cells has opened the possibility of DNA vaccination [2]. DNA vaccination for malaria has many advantages including: (1) the ease of production and stability of a vaccine; (2) the ability to immunise against many antigens using a mixture of plasmids; and (3) the ability to efficiently elicit cytotoxic and helper T-cells and good antibody production. Gene transfer through the introduction of mRNA is also possible, leading to the concept of RNA vaccination [3]. Although mRNA is difficult to prepare and less stable than plasmid DNA, it has the advantage that chromosomal integration of the introduced nucleic acid, which is a possible drawback with DNA vaccines, does not occur.

Because of their accessibility to antibodies, proteins on the merozoite surface are candidate molecules for developing a vaccine against asexual blood stages of the malaria parasite. Monoclonal antibodies (mabs) against a 45–50 kDa *Plasmodium falciparum* merozoite surface antigen, MSA-2 (alternatively termed GYMSSA or PfMSP-2) [4,5], are reported to inhibit parasite growth in vitro [6,7]. Some mabs against a 185–200 kDa merozoite surface antigen (termed MSA-1, PMMSA or PfMSP-1) similarly inhibit parasite growth [8], although other mabs can prevent the inhibition [9]. Antibodies against MSA-1 [10–12] and MSA-2 [13] in endemic area persons have also been associated with protection against the clinical symptoms of falciparum malaria.

MSA-2 occurs as two major allelic types that differ chiefly in a central region composed of repetitive sequences and the associated flanking regions. The N-terminal 42 and C-terminal 75 amino acids are largely conserved in different parasite isolates [14–16]. Recombinant MSA-2 protein, derived from the 3D7 *P. falciparum* isolate and produced in *Escherichia coli* has been recently used, together with a recombinant circumsporozoite protein, in a combined Phase I and Phase II clinical trial. The proteins were reported to be safe and immunogenic, but there was no evidence of protective immunity against malaria in the vaccinees [17]. We describe here the cloning and expression of MSA-2 from the 3D7 *P. falciparum* isolate in mammalian cells and the antibody response to MSA-2 based DNA and RNA vaccines in rabbits.

Immunisation with intact MSA-1 or its C- and N-terminal fragments protected *Aotus* and *Saimiri* monkeys against falciparum malaria [18–21]. Protection in these animal models was related to high titre antibodies. Antibodies to a C-terminal 19 kDa proteolytically processed fragment of MSA-1, which is carried into RBC by invading merozoites, are particularly important for protective immunity [8]. However an 11 aa sequence located close to the N-terminus of the mature protein is a constituent of SPf66, a widely publicised malaria vaccine that reportedly protects against malaria [22]. Other reports also suggest a role for the N-terminus of MSA-1 in eliciting protection [11,12,19,20]. MSA-1 is essentially coded for by dimorphic alleles capable of limited genetic exchange [23]. The MSA-1 sequence of >1700 aa has been divided into 17 blocks composed of regions that are: (1) conserved; (2) semi-conserved and; (3) variable between the two alleles. We cloned two fragments of 1326 and 1386 bp, from the 5′ end of the MSA-1 gene, which differed in the presence or absence of the native signal peptide sequence, into mammalian expression vectors for experimental DNA immunisation of rabbits. The cloned region of MSA-1 comprises blocks 1–5 plus a portion of block 6 [23]. Blocks 1, 3 and 5 are conserved regions while blocks 2 and 4 are different in the two alleles [23]. Block 2 is composed of the repeated sequence SGASAQ in the 3D7 isolate that fits the consensus repeat sequence SXXSXX for block 2.

Immunisation with synthetic peptides containing known B-cell epitopes from MSA-1 and MSA-2 coupled to diphtheria toxoid as a carrier molecule in a Phase I clinical trial, showed good antibody formation [24]. However, an unacceptable level of

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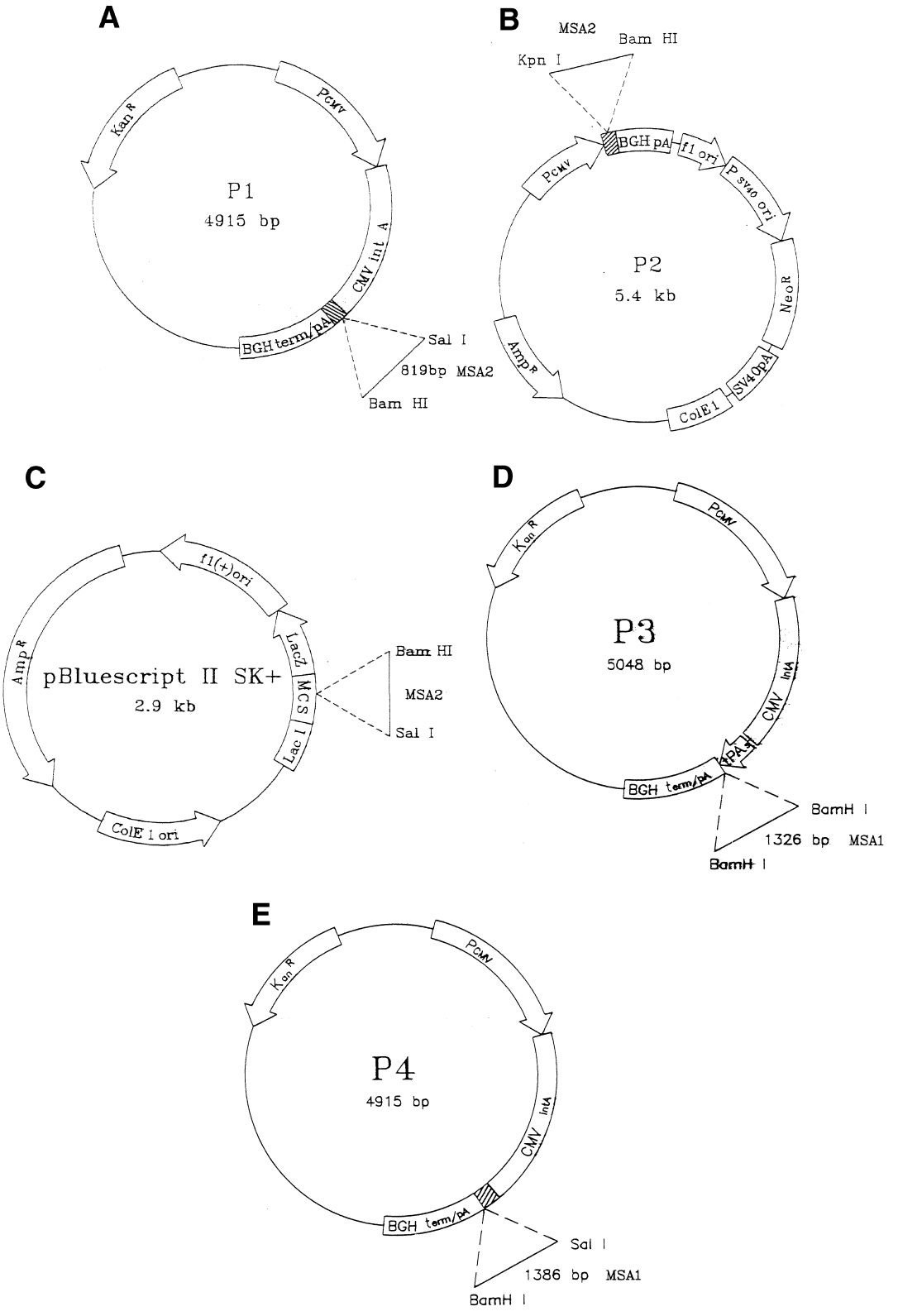
Fig. 1. (A) Map of plasmid P1 derived by cloning the MSA-2 coding sequence into VR1012. (B) Map of plasmid P2 derived by cloning the MSA-2 coding sequence into pcDNA3 (Invitrogen, USA). (C) Map of plasmid BKG which was produced by cloning the MSA-2 coding sequence into pBluescript SKII$^+$. (D) Map of plasmid P3 derived by cloning a 1326 bp MSA-1 sequence into VR1020. (E) Map of plasmid P4 derived by cloning a 1386 bp MSA-1 sequence, including the signal peptide sequence, into VR1012.


A
P1
4915 bp
Kan R
PCMV
CMV int A
BGH term/pA
Sal I
819bp MSA2
Bam HI
B
MSA2
Bam HI
Kpn I
BGH pA
f1 ori
PCMV
P SV40 ori
P2
5.4 kb
NeoR
SV40pA
ColE1
AmpR
C
pBluescript II SK+
2.9 kb
f1(+)ori
LacZ
MCS
Lac I
AmpR
ColE 1 ori
Bam HI
MSA2
Sal I
D
P3
5048 bp
Kan R
PCMV
CMV intA
BPA pA
BGH term/pA
BamH I
1326 bp MSA1
BamH I
E
P4
4915 bp
Kan R
PCMV
CMV intA
BGH term/pA
Sal I
1386 bp MSA1
BamH I

type III (Arthus) hypersensitivity to the toxoid was observed in the immunised individuals [24] and this precluded a subsequent Phase II trial. Experimental nucleic acid immunisation with the two merozoite proteins was expected to overcome some of the disadvantages of the peptide–diphtheria toxoid system.

2. Materials and methods

2.1. Cloning of MSA-2 gene fragments into mammalian expression vectors

DNA isolated from 3D7 parasites by binding to Sephaglas (Pharmacia) [14], was used as a template for PCR amplifying the 819 bp coding sequence of the MSA-2 gene. The forward and reverse primers employed were AAAGTCGACATGAAGGTAATTAAAAC (designed to yield a *Sal*I site) and ACAAAGCTTGGATCCTTATATGAATATGGCAAAAGATA (designed to yield a *Bam*HI site), respectively. The amplified gene was cloned into the *Sal*I–*Bam*HI site of the eukaryotic expression vector VR1012 [25,26] to yield plasmid P1 (Fig. 1A). Expression in P1 is under the control of a human cytomegalovirus (CMV) immediate-early gene promoter and a CMV intron A enhancer. P1 also carries a bovine growth hormone (BGH) termination/poly A signal 3′ to the cloned gene fragment. Vector VR1012 (and VR1020 described below) kindly provided by Drs. R.C. Hedstrom and S.L. Hoffman of the Naval Medical Research Institute, MD, USA, had the ampicillin resistance gene found in pUC18-derived plasmids replaced by a kanamycin resistance gene. The PCR amplified MSA-2 gene was also cloned into the *Sal*I–*Bam*HI site of the phagemid pBluescript IISK$^+$ (Stratagene, USA). pBluescript II carries T7 and T3 RNA polymerase promoter sequences flanking the multiple cloning site. The sequence of the cloned gene was confirmed by dideoxynucleotide sequencing [27]. The MSA-2 gene was excised from pBluescript IISK as a *Kpn*I–*Bam*H I fragment and cloned into the *Kpn*I–*Bam*HI site of the eukaryotic expression vector pcDNA3 (Invitrogen, USA) to yield plasmid P2 (Fig. 1B). Expression of the cloned gene in P2 is under the control of a CMV promoter and BGH termination/polyadenylation signals.

2.2. Production of MSA-2 mRNA

pBluescript SKII$^+$ carrying MSA-2 (Fig. 1C) was cleaved with *Bam*HI and *Not*I within the polylinker site of the phagemid, and a double-stranded deoxyoligonucleotide containing the coding strand sequence $(A)_{35}$ flanked by *Bam*HI and *Not*I sites cloned into the 3′ end of the MSA-2 gene. The sequence of the two oligonucleotides used were GATCC$(A)_{35}$GC for the coding strand and GGCCGC$(T)_{35}$G for the template strand. Clones of phagemid containing the poly A tail were selected by the presence of a 230 bp *Not*I–*Xba*I fragment. The phagemids carrying the MSA-2 gene with and without the poly A tail were termed BKA and BKG, respectively. Approximately 40 μg mRNA were transcribed, using the T7 Cap-Scribe reagents (Boehringer, Germany) from 0.5 μg of each of the two phagemids linearised by *Not*I digestion, and then purified by ethanol precipitation according to the manufacturer's instructions. The products incorporated $m^7G(5')ppp(5')G$ caps for stability and efficient translation. On analysis in denaturing agarose gels [27], the RNA transcribed from BKG migrated as expected of a 839 nucleotide species, while BKA RNA migrated as a slightly larger molecule corresponding to an expected size of 862 nucleotides.

2.3. Cloning of MSA-1 gene fragments into mammalian expression vectors

A 1386 bp fragment of MSA-1 DNA coding for a 462 aa N-terminal polypeptide including the initiating methionine was PCR amplified using the forward primer RR96-5 and the reverse primer RR96-20. A 1326 bp DNA fragment coding for a 442 aa N-terminal polypeptide, where 20 aa corresponding to the signal peptide and an additional valine were absent, was similarly PCR amplified with RR96-21 as the forward primer and RR96-20 as the reverse primer. RR96-5 incorporates a site for *Sal*I, while RR96-20 and RR96-21 incorporate sites for *Bam*HI. The sequences of the primers were: RR96-5, AAAGTCGACATGAAGATCATATTCTTTTTATGTTCATTTC; RR96-20, TAATGGATCCTTCGTGTTCCTCGATTTT; and RR96-21, CCCGGATCCACACATGAAAGTTATCAAG.

The gene fragment coding for the MSA-1 protein without the signal peptide was cloned into the *Bam*HI site of the eukaryotic expression vector VR1020 to yield plasmid P3 (Fig. 1D). A human tissue plasminogen activator (tPA) signal peptide sequence is incorporated 5′ to the cloned gene in P3. The MSA-1 gene fragment bearing the sequence for the native signal peptide was cloned into VR1012 as a *Sal*I–*Bam*HI fragment to yield plasmid P4 (Fig. 1E). VR1012 differed from VR1020 chiefly in lacking the tPA signal sequence. P4 clones were selected from minipreps [27] by the presence of the appropriate size *Sal*I–*Bam*HI fragment. The selection of recombinant VR1020 colonies was aided by a digoxigenin-tagged MSA-1 probe prepared by random primed labelling of the PCR amplified MSA-1 DNA with digoxigenin labelled dUTP according to the manufacturer's instructions (DIG labelling kit, Boehringer, Germany) and alkaline phosphatase conjugated anti-digoxigenin antibodies [28]. P3 clones containing the inserted DNA fragment in the correct orientation were subsequently selected from recombinant VR1020 clones by the detection of the appropriate size *Hin*dIII digestion fragments from miniprep DNA.

2.4. Preparation of plasmid DNA for immunisation

Clones grown in DH5α cells were used to isolate plasmid DNA on Qiagen columns according to the manufacturer's instructions (Qiagen, Germany). Plasmid DNA was dissolved in water, the concentration and purity determined by measuring absorbance at 260 and 280 nm, and stored in aliquots at approximately 1 mg ml^{-1} at −20°C.

2.5. Antibodies to MSA-1 and MSA-2

The IgG1 mouse mab 12.2 reacting with the allele-specific block 2 repeats found at the N-terminus of 3D7 MSA-1 and IgG1 mabs 12.3 and 12.7 reacting with the allele-specific region of 3D7 MSA-2 were kindly provided as ascites fluids by Dr. J. McBride of the University of Edinburgh. Rabbit antiserum to MSA-2 was also raised by immunisation with a purified MSA-2–glutathione *S*-transferase fusion protein.

2.6. Expression of MSA-1 and MSA-2 in Cos-1 cells

Cos-1 SV40 transformed monkey cells (ATCC CRL-1650) grown in RPMI 1640 (Sigma) containing 10% foetal calf serum, were transfected with 2–8 μg plasmid DNA using the *N*-[1-(2,3-D dioleoyloxy) propyl]-*N*,*N*,*N*-(trimethylammonium) methyl sulphate (DOTAP) transfection reagent (Boehringer, Germany) according to the manufacturer's instructions. Approximately 12% of cells were transfected by this procedure when the transfection efficiency was determined by using an eukaryotic expression vector carrying β-galactosidase (pCH110, Pharmacia) and staining with X-gal (R. Ramasamy, unpublished data). After 48 h of culture, the transformed Cos cells were washed and lysed by adding Laemmli sample buffer [29]. After boiling at 100°C, the lysate was repeatedly passed through a 27-gauge needle to shear DNA and aliquots were used for SDS-PAGE [29], transferred to nitrocellulose [30] and probed with specific mabs in Western blots. Cos cells similarly transfected in parallel with non-recombinant expression vectors were used as a control.

2.7. Expression of MSA-2 in the vaccinia/T7 transient expression system [31]

HeLa cells grown to 80% confluence in 25 cm^2 tissue culture flasks were washed in 0.01 M phosphate buffered saline, pH 7.2 (PBS) and infected with a recombinant vaccinia virus strain (vTF7-3) carrying the T7 RNA polymerase gene at 30 pfu per cell for 30 min at 37°C. The infected cells were then transfected with 4 or 20 μg BKG DNA mixed with 27 μg DOTAP. After 24 h, the HeLa cells were collected, washed and then lysed in Laemmli sample buffer and used for Western blots as described above.

2.8. DNA immunisation of rabbits against MSA-2 with plasmids P1 and P2

P1 and P2 were each used separately to intradermally immunise two New Zealand white rabbits. Additionally, P1 was used to intramuscularly immunise two other rabbits. Each rabbit was immunised four times at 3 week intervals. The rabbits were ear bled before immunisation to yield the prebleed serum and then 3 weeks after each immunisation. For each rab-

bit immunisation, 25 μg DNA was mixed with DOTAP liposomes according to the manufacturer's instructions (Boehringer, Germany). Immunisation was carried out intradermally at multiple sites using washed and sterilised commercial tuberculin applicators. Intramuscular injections were performed at two sites per rabbit using a sterile 1 ml disposable syringe. Rabbits were immunised with non-recombinant plasmids VR1012 and pcDNA3 in parallel as controls. Rabbits T1 and T2, immunised intramuscularly with P1, were additionally bled 11 weeks after the fourth immunisation and rabbit T5, immunised intradermally with P1, was bled 14 weeks after the fourth immunisation to test for persistence of antibodies. The care and use of animals were according to WHO guidelines (WHO88.1).

2.9. RNA immunisation of rabbits against MSA-2 with BKA and BKG

Forty μg of BKA mRNA was mixed with 125 μg DOTAP in a volume of 300 μl sterile HBS as described above and 230 μl injected intramuscularly into a rabbit at two sites. The remaining 70 μl of the mRNA/DOTAP mix was inoculated intradermally at multiple sites using a tuberculin applicator. Another rabbit was injected with BKG mRNA in an identical manner. Three immunisations were performed at 3 week intervals. The rabbits were ear bled before immunisation to yield the prebleed serum and then 14 days after each immunisation. A final bleed was obtained 15 weeks after the third immunisation to test the persistence of antibodies in the rabbit injected with BKA mRNA.

2.10. DNA immunisation of rabbits against MSA-1 with plasmids P3 and P4

Each plasmid was injected into two New Zealand white rabbits at 3 week intervals. The rabbits were ear bled before immunisation to yield preimmune serum and then 3 weeks after each immunisation. For each rabbit immunisation, 25 μg DNA was complexed with DOTAP. Due to the limited availability of DOTAP and other reagents, only one protocol for immunisation with the two plasmids was possible. The first three immunisations were done by intramuscular injection at two sites per rabbit. The fourth immunisation was performed intradermally at multiple sites using a washed and sterile commercial tuberculin applicator with half the DOTAP–DNA complexes (12.5 μg DNA). The remainder was injected intramuscularly into the same rabbit. Sera were also obtained 9 and 48 weeks after the fourth immunisation from the two more strongly responding rabbits injected with each construct (rabbits 2 and 4) to test for the persistence of antibodies.

2.11. Immunofluorescence assay (IFA) and Western blots on parasite lysate

IFA and Western blots were performed on late stage 3D7 parasites, prepared by gelatine sedimentation, as described previously [4]. Antibody titres were determined using serial 10-fold dilutions of sera or IgG preparations.

2.12. Enzyme-linked immunosorbent assay (ELISA) on MSA-1 and MSA-2 peptides

Peptide P109 VTHESYQELVKKLEALEDAC containing the N-terminal sequence of the mature MSA-1 protein with an added C-terminal cysteine, was used to assay antibodies to MSA-1 as previously described [32]. Two peptides derived from the conserved N- and C-terminal regions of the MSA-2 molecule were used similarly in the ELISA for MSA-2 antibodies. The peptides were P101 (NESKYSNTFINNAYNMSIRC, residues 23–40 of the FC27 isolate sequence with an additional C-terminal cysteine) and P103 (RNNHPQNTSDSQKEATDGNKC, residues 207–226 of the FC27 isolate sequence with an alanine replacing a cysteine at position 221 of the native sequence and a C-terminal cysteine introduced for coupling purposes).

2.13. Parasite growth inhibition assay

The 3D7 isolate of *P. falciparum* from Dr. D. Walliker, University of Edinburgh was used for measuring rabbit IgG antibody-mediated growth inhibition by microscopy and [^{3}H]hypoxanthine incorporation [33,34]. IgG was prepared from rabbit sera preabsorbed with human RBCs, by precipitation with 50% ammonium sulphate and exhaustive dialysis into RPMI 1640. The IgG content as estimated by

SDS-PAGE analysis [29] was 50%. Protein concentration was determined by a Lowry assay [35]. In other growth invasion experiments, rabbit sera preabsorbed with human RBCs were used at 10% v/v to replace human serum in the culture medium. Preimmune rabbit IgG and sera were used as controls.

3. Results

3.1. Expression of MSA-2 in Cos and HeLa cells

Transfection of Cos cells with P1 and P2 showed that the MSA-2 was only weakly detected in Western blots probed with the MSA-2 specific antibodies. Synthesis of MSA-2 in the vaccinia/T7 polymerase transient expression system, which is reportedly two orders of magnitude more efficient than the conventional system [31], was therefore attempted. HeLa cells infected with recombinant vaccinia virus expressing T7 RNA polymerase and then transfected with 4 μg BKG in 27 μg DOTAP, synthesised MSA-2 that was readily detected in Western blots (Fig. 2). The use of a higher DNA to DOTAP ratio in the transfection assay reduced the efficiency of transfection/expression. The MSA-2 synthesised in HeLa cells migrated as a 50 kDa protein on SDS-PAGE. The observed size of the MSA-2 synthesised in HeLa cells is similar to the apparent M_r of recombinant MSA-2 produced in *Escherichia coli* (Fig. 2) and parasite synthesised MSA-2 (Figs. 3–5). The observed M_r is almost double the molecular mass of 27 972 predicted for the 273 residue protein.

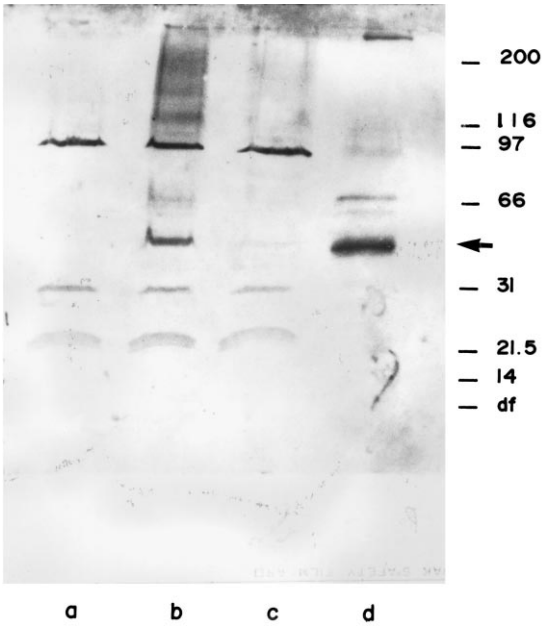


Fig. 2. Western blot showing the synthesis of MSA-2 in HeLa cells transfected with pBluescript SKII$^+$ carrying the MSA-2 gene (BKG) after infection of the cells with recombinant vaccinia coding for T7 RNA polymerase. One million vaccinia infected HeLa cells were transfected with: lane a, 4 μg control pBluescript SKII; lane b, 4 μg BKG; lane c, 20 μg BKG and 10% of the lysates separated on 10% gels. Lane d, 100 ng recombinant MSA-2 protein produced in *E. coli*. Mab 12.3 was used as the first antibody for probing the blot. The migration positions of marker proteins are indicated in kDa and of MSA-2 by an arrow. df, dye front. The prominent 90, 30 and 23 kDa bands in lanes a–c probably represent HeLa cell or vaccinia proteins recognised by antibodies in the ascites fluid containing mab 12.3.

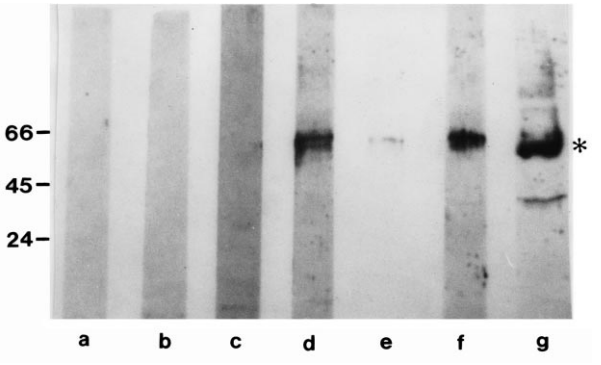


Fig. 3. Antibody production after intramuscular injection of rabbit T2 with P1 DNA. Western blots of 3D7 parasite lysates were probed with 10^{-2} dilution of serum obtained 3 weeks after: lane a, one injection; lane b, two injections; lane c, three injections; and lane d, four 4 injections. Lane e, parasites probed with 10^{-3} dilution of the serum obtained 3 weeks after the four the injection; lane f, parasites probed with 10^{-2} dilution of serum obtained 11 weeks after the fourth injection; lane g, parasites probed with 1:500 dilution of antiserum to recombinant MSA-2 protein. Migration positions of marker proteins are indicated in kDa and of MSA-2 by an asterisk.

3.2. Antibody response to DNA and RNA immunisation with MSA-2

Intramuscular injection of P1 DNA into rabbits led to the production of antibodies to MSA-2 detectable by Western blotting (Fig. 3). Antibodies were also produced in rabbits after intradermal inoculation of P1 DNA (Fig. 4) and P2 DNA (Fig. 5). Parasite synthesised MSA-2 was detected as a broad and sometimes split band in Western blots (e.g. Fig. 3,

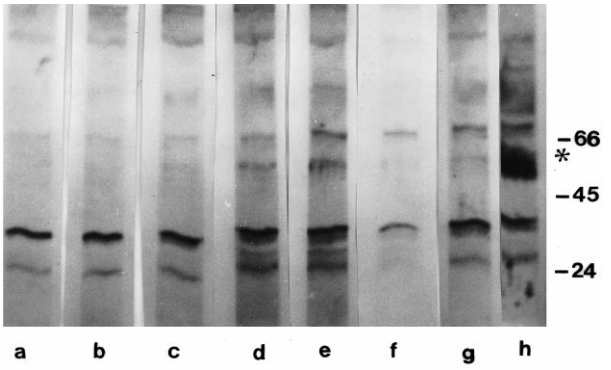


Fig. 4. Antibody production after intradermal inoculation of rabbit T5 with P1 DNA. Western blots of 3D7 parasite lysates were probed with 10^{-2} dilution of serum obtained: lane a, before immunisation; lane b, 3 weeks after one inoculation; lane c, 3 weeks after two inoculations; lane d, 3 weeks after three inoculations; lane e, 3 weeks after four inoculations. Lane f, parasites probed with 10^{-3} dilution of the serum obtained 3 weeks after four inoculations; lane g, parasites probed with 10^{-2} dilution of serum obtained 14 weeks after the fourth inoculation; lane h, parasites probed with 10^{-3} dilution of antiserum to recombinant MSA-2 protein. Migration positions of marker proteins are indicated in kDa and of MSA-2 by an asterisk.

lane d and Fig. 4, lane h). Antibodies were also clearly detected by immunofluorescence and weakly by Western blotting after immmunisation of rabbits with BKA mRNA. The reaction of sera from BKG mRNA inoculated rabbit with parasites was only detectable weakly by IFA at 10^{-1} dilution.

None of the sera from the DNA or RNA immunised rabbits, when tested at dilutions of 10^{-1} to 10^{-4}, reacted with the two peptides from the conserved N- and C-termini of MSA-2 in an ELISA. Antibodies were detectable by IFA after three immunisations in all rabbits subject to DNA and RNA vaccination with MSA-2. The IFA pattern observed on late stage schizonts resembled a bunch of grapes which is characteristic of reaction with antigens, such as MSA-2, present on the merozoite surface [4]. No IFA or Western blot reactions were seen with the sera of control rabbits immunised with non-recombinant plasmids or preimmune sera from the rabbits immunised with P1, P2 or BKA. The results of antibody assays performed on the immune rabbit sera are summarised in Table 1.

Table 1
Antibody response to MSA-2 after DNA and RNA immunisation

Immunogen (route of inoculation)	Rabbit	IFA titre after 3rd immunisation	Western blot titre after 4th immunisation	Characteristics of Western blots
P1 (intramuscular)	T1	10^{-2}	10^{-2}	Ab first detected after 3 immunisations and at 10^{-2} dilution 11 weeks after 4th immunisation
P1 (intramuscular)	T2	10^{-2}	10^{-3}	Ab first detected after 3 immunisations and at 10^{-2} dilution 11 weeks after 4th immunisation
P1 (intradermal)	T5	10^{-2}	10^{-3}	Ab first detected after 2 immunisations and at 10^{-2} dilution 14 weeks after 4th immunisation
P1 (intradermal)	T6	10^{-2}	10^{-3}	Ab first detected after 2 immunisations
P2 (intradermal)	T7	10^{-2}	10^{-2}	Ab first detected after 3 immunisations
P2 (intradermal)	T8	10^{-2}	10^{-2}	Ab first detected after 3 immunisations
BKA mRNA	BKA	10^{-2} (also detected at 15 weeks after 3rd immunisation at 10^{-2} dilution)	10^{-2} (after 3rd immunisation)	Ab first detected after 3 immunisations. Weak reaction. Only 3 immunisations performed
BKG mRNA	BKG	10^{-1} (weak)	negative (after 3rd immunisation)	Only 3 immunisations performed

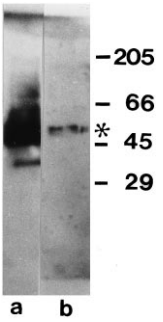


Fig. 5. Antibody production in rabbit T7 after intradermal inoculation with P2 DNA. Western blots of 3D7 parasite lysates were probed with: lane a, antiserum to recombinant MSA-2 protein at 1:500 dilution; and lane b, T7 serum at 10^{-2} dilution obtained 3 weeks after the third inoculation. Migration positions of marker proteins are indicated in kDa and of MSA-2 by an asterisk.

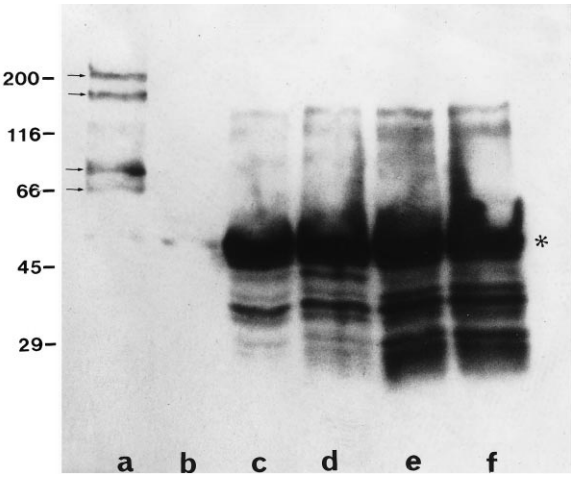


Fig. 6. Western blot of: lane a, late stages of *P. falciparum* 3D7 isolate, and Cos cells transfected with lane b, 4 μg of control plasmid pcDNA3; lane c, 4 μg plasmid P4; lane d, 8 μg P4; lane e, 4 μg P3; and lane f, 8 μg P3. The blot was probed with mab 12.2 against MSA-1. The migration position of intact MSA-1 and its major fragments recognised by mab 12.2 in the parasite lysate (lane a) are indicated by arrows. The migration position of the MSA-1 fragment synthesised in Cos cells is indicated by an asterisk. The migration positions of the different molecular weight markers are also shown.

None of the sera from immunised rabbits used at 10% v/v in cultures, or IgG from immunised rabbits T1, T7 and BKA at concentrations in the range 2 μg ml^{-1} to 2 mg ml^{-1} in cultures, significantly inhibited parasite growth compared to preimmune sera or IgG from the same rabbits in a [^{3}H]hypoxanthine incorporation assay. In one experiment, for example, at 2 mg ml^{-1} T1 IgG obtained after four injections gave 14566 ± 1028 (cpm ± S.E.) while preimmune T1 IgG gave 15520 ± 1429 (cpm ± S.E.) at the same concentration incorporation into parasites.

3.3. Expression of MSA-1 in Cos cells

Cos-1 cells transfected with P3 and P4 synthesised a protein of the expected M_r of approximately 50 kDa that reacted with the anti MSA-1 mab 12.2. in Western blots (asterisk in Fig. 6). Smaller proteins reacting with the mab in P3 and P4 transfected cells are probably degradation products (Fig. 6).

3.4. Antibody response to DNA immunisation with MSA-1

The results of antibody assays performed with immune sera obtained after four immunisations with P3 and P4 are summarised in Table 2. Sera obtained

Table 2
Antibody response to MSA-1 after DNA immunisation

Rabbit (immunogen)	ELISA titre on P109 after four immunisations	IFA titre after four immunisations
No. 1 (P3)	negative	10^{-1}
No. 2 (P3)	10^{-2}	10^{-2} (Ab first detected at 10^{-1} dilution after 2nd immunisation. Maintained for 9 weeks after 4th immunisation)
No. 3 (P4)	negative	10^{-1}
No. 4 (P4)	10^{-2}	10^{-3} (Ab first detected at 10^{-1} dilution after 2nd immunisation. Maintained for 9 weeks after 4th immunisation)

from one rabbit of each category after four immunisations, showed a detectable antibody response to MSA-1 by ELISA with peptide P109. Sera from all the rabbits after four immunisations gave a clear IFA fluorescence pattern on late schizonts resembling a bunch of grapes that is indicative of reaction with the merozoite surface [4]. However, the same sera only reacted weakly with MSA-1 in Western blots (data not shown). IFA antibody titres, which were maintained for at least 9 weeks after the fourth immunisation in the two more strongly responding rabbits (nos. 2 and 4), declined to background levels by 48 weeks. Sera from rabbits immunised with control plasmids or the preimmune sera from the immunised rabbits did not react with MSA-1 in any of the antibody assays.

Immune rabbit sera at 10% v/v or IgG preparations at concentrations in the range of 1 μg ml^{-1} to 1 mg ml^{-1} did not inhibit *P. falciparum* growth over 48 h as determined by either microscopy or the [^{3}H]hypoxanthine incorporation assay. For example, in one experiment, rabbit no. 4 preimmune IgG at 1 mg ml^{-1} yielded 11690 ± 816 (cpm ± S.D.) while IgG from the same rabbit after four immunisations at 1 mg ml^{-1} yielded 13988 ± 2561 (cpm ± S.D.) in the [^{3}H]hypoxanthine incorporation assay.

4. Discussion

Transient transfection assays were first used to demonstrate that the cloned merozoite proteins are synthesised in primate cells. The 462 aa N-terminal MSA-1 fragment was better expressed than the 273 aa MSA-2 protein when the corresponding genes were cloned into VR1012 and transfected into Cos cells. The explanation for this is not clear, but may be related to characteristics of the two gene or protein fragments. For example, the MSA-2 molecule carries native signals for translocation into the endoplasmic reticulum (ER) and the addition of a membrane anchoring glycosylphosphatidyl inositol (GPI) moiety, while the MSA-1 fragment only carries the ER signal. The results also show that MSA-1 polypeptide carrying the human tPA signal peptide (in VR1020) or the native signal peptide (in VR1012) was about equally expressed in Cos cells. Although the general structural features of signal peptides are conserved from bacteria to man, few malaria parasite genes coding for exported or membrane proteins have previously been tested for protein expression in primate cells. More detailed studies, including determining the definitive subcellular location of MSA-1 and 2 synthesised in primate cells, are necessary for establishing whether parasite signal sequences for ER export or the addition of a GPI moiety are recognised in these cells.

The anomalous migration of MSA-2 in SDS-PAGE is not due to glycosylation or the attachment of the GPI moiety since recombinant MSA-2 produced in *E. coli* also migrated as a 50 kDa protein on SDS-PAGE. The presence of repeats and flanking sequences with unusual amino acid composition [1] and the acidic nature of MSA-2 (predicted p*I* 5.25) may be responsible for the retarded migration of MSA-2 in SDS-PAGE. A recombinant protein containing the proline rich repetitive region of the *P. berghei* thrombospondin related adhesive protein showed similar anomalous migration on SDS-PAGE [36]. Possible microheterogeneity in glycosylation and the presence of MSA-2 molecules with and without other post-translational modifications in parasites may be further reasons for the broad and sometimes split band of MSA-2 observed in Western blots of parasite lysates.

The production of antibodies in rabbits in response to injection of the plasmids establish the expression of MSA-2 and the MSA-1 fragment in rabbit tissues under the control of the CMV promoter and intron A enhancer. The promoter of the human CMV major immediate early gene and the intron A enhancer from the same gene have proved to be effective in controlling protein expression in different cell types [25,26,37]. However, expression of MSA-2 was also observed in the absence of the intron A enhancer in these studies. Intradermal inoculation with a tuberculin applicator was about as effective as intramuscular injection of plasmid DNA for generating rabbit antibodies to MSA-2. Plasmid DNA introduced intradermally may transfect skin Langerhans cells which are particularly effective antigen presenting cells. Introduction of mRNA bound to DOTAP liposomes into rabbit tissues also proved immunogenic, but the presence of an $(A)_{35}$ tail in the mRNA enhanced antibody formation. This is probably due to the greater stability and/or transla-

tion efficiency conferred by the poly A tail. Injection of DNA and capped and polyadenylated mRNA without cationic liposomes has also been shown to efficiently transfect muscle tissue [3]. However, injection of DNA complexed with cationic liposomes may result in more widespread antigen expression if the complexes enter the bloodstream [38].

Parameters, such as the amount of nucleic acid inoculated, number of doses and the interval between doses, the presence of immunostimulatory DNA sequences in the vector [39] and the route of immunisation, influence the efficacy of nucleic acid vaccination and require investigation before clinical trials are warranted. Safety concerns for eventual human use, e.g. integration of injected DNA into chromosomes, induction of tolerance or autoimmunity and production of anti-DNA antibodies also need to be addressed, but the risks appear negligible at present [40]. The long-term maintenance of antibody levels in the DNA immunised rabbits is advantageous for nucleic acid vaccination and may be due to the persistence of the plasmid in transfected cells [41]. The persistence of antibodies 15 weeks after inoculation of BKA mRNA indicates the potential for RNA vaccination. The basis for the comparable stability of antibody titres after mRNA and DNA mediated MSA-2 vaccination is not clear. It is possible that, after either RNA or DNA immunisation, MSA-2 is only slowly degraded in the transfected cells or that adequate MSA-2 accumulates on follicular dendritic cells in the form of stable immune complexes to stimulate antibody production for several months. The results also show that, despite poorer relative expression in Cos cells, the P1/P2 encoding MSA-2 elicited better antibody titres in rabbits than P3/P4 encoding a considerably larger MSA-1 protein fragment. The presence of a greater proportion of repetitive sequence in the MSA-2 protein, possible instability of the MSA-1 protein fragment and different cellular localisation of the two proteins may be some of the factors that influence immunogenicity of the two DNA vaccines.

Considerably greater immunisation efficiency was reported with biolistic delivery of gold particles coated with DNA into the epidermis [42]. Antibody titres $>10^{-3}$ and cytotoxic T-lymphocyte responses to influenza nucleoprotein were elicited by as little as 16 ng of DNA given in a single dose to mice by biolistic delivery [42]. Biolistic delivery does not require cationic lipids such as DOTAP, and the use of a hand-held gene gun to deliver particle coated DNA is more appropriate for vaccinating large populations [42]. VR1012 and VR1020 where problematic sequences present in pcDNA3, such as an SV40 origin and the ampicillin resistance gene, have been deleted are suitable for human DNA vaccination in this way. The biolistic delivery of mRNA for immunisation also merits investigation.

Nucleic acid immunisation is a particularly effective means of generating cytotoxic T-cell responses since the protein of interest is synthesised in the cytoplasm of the transfected cell and can then be efficiently presented by MHC class I molecules on the surface. Since cytotoxic T-cells are effective against malaria, parasite infected hepatocytes, the use of nucleic acid vaccination is particularly appropriate for targeting these stages, and studies in rodent malaria indicate its usefulness [43]. In addition to liver stage specific proteins, MSA-1 and MSA-2 synthesised in infected hepatocytes may provide targets for cytotoxic T-cells and T_h1-cells that are known to be effective in eliminating infected hepatocytes [1,43]. This possibility requires investigation. T_h-cells and antibodies are, however, more important for controlling blood stage malaria infection. Antibodies may act to block RBC recognition or invasion by merozoites [6,7], cause complement mediated lysis of merozoites [34], or promote antibody-dependent, cell-mediated cytotoxicity against parasites [44–46]. *P. falciparum* cultures do not contain leucocytes and therefore only the first two mechanisms are potentially operative in vitro. Inadequate antibody concentration or affinity may be responsible for the lack of growth inhibition observed in the experiments. This is an important consideration since merozoites invade RBC rapidly. However, we have observed that rabbit antibodies to a recombinant MSA-2 protein, with Western blotting and IFA titres of 10^{-6}, do not inhibit *P. falciparum* growth in vitro and instead promote multiple invasion of RBCs at 2–200 μg ml^{-1} of total IgG (R. Ramasamy and S. Yasawardena, unpublished observations). The ELISA results imply that the antibody response to MSA-2 in our experiments is directed predominantly against the allele-specific or non-conserved regions of the molecule. Repetitive sequences are prominent in the allele specific regions of MSA-2,

and also occur in MSA-1. The repeats may help the parasite escape host immunity by: (1) exhibiting sequence polymorphism; (2) preventing the normal affinity and isotype maturation of the immune response; (3) functioning possibly as B-cell superantigens; (4) generating predominantly thymus independent antibody responses; and (5) acting as a sink for binding protective antibodies. The evidence for such immune evasion mechanisms have been reviewed elsewhere [1]. Approximately a quarter of the MSA-1 sequence at the N-terminus, and including the repeat region, was used for immunisation in the present study. The binding to merozoites of mabs directed against the C-terminal 19 kDa MSA-1 fragment that inhibit proteolytic processing of MSA-1 and also merozoite invasion, can be prevented by certain other mabs against the 19 kDa fragment [9] and human polyclonal antibodies against the more N-terminal region of MSA-1 [47] that do not themselves inhibit merozoite invasion or proteolytic processing. The ability of the parasite to elicit such blocking antibodies may constitute another mechanism for avoiding host immunity. However, other studies indicate a role for the MSA-1 N-terminus in protective immunity [11,12,19,20,22]. The $>90\%$ inhibition of parasite growth observed previously with IgG mabs to MSA-2 at 125 μg ml^{-1} [5,6] may be the result of high concentration and affinity and/or specificity for critical epitopes. It is notable in this context that IFA antibody titres in vaccinees receiving three injections of recombinant 3D7 MSA-2 in a Phase II clinical trial, who were subsequently shown not to be protected, were in the range 1/80 to 1/320 [17]. The development of vaccines based on *P. falciparum* merozoite surface proteins will need to address all these issues.

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