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Immunogenicity of a malaria parasite antigen displayed by *Lactococcus lactis* in oral immunisations

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Abstract

A putative protective protein from *Plasmodium falciparum* merozoites, MSA2, was expressed in two different ways on the cell surface of the Gram-positive food-grade bacterium, *Lactococcus lactis*. The first display format exploits an LPXTG-type anchoring motif of the lactococcal proteinase PrtP to covalently anchor MSA2 to the genetically modified producer cells. In a second display format, MSA2 was fused to the peptidoglycan-binding domain (Protein Anchor) of the lactococcal cell wall hydrolase AcmA and was non-covalently rebound to the surface of non-genetically modified, non-living high-binder *L. lactis* cells, termed Gram-positive enhancer matrix (GEM) particles. The *L. lactis* recombinants carrying covalently bound MSA2 were used to immunise rabbits through nasal and oral routes. The highest levels of IgG antibodies reacting with near-native MSA2 on merozoites was elicited by oral administration. Intestinal antibodies to MSA2 were produced only after oral immunisation. MSA2-specific T_h-cell activation could be demonstrated. Based on these results, the immunogenicity in oral immunisations of MSA2, bound non-covalently to non-genetically modified *L. lactis* GEM particles, was compared with MSA2 that was bound covalently to genetically modified *L. lactis*. These two forms elicited similar titres of serum antibodies. The results illustrate the potential of using non-genetically modified *L. lactis* as a safe vaccine delivery vehicle to elicit systemic antibodies, thereby avoiding the dissemination of recombinant DNA into the environment.

Keywords: Surface display; *Lactococcus lactis*; Gram-positive enhancer matrix (GEM) particles; *Plasmodium falciparum*; Merozoite surface antigen 2 (MSA2); Mucosal vaccines

1. Introduction

Nasal and oral vaccination is preferable to injections from the point of view of safety, ease of administration and compliance. Mucosal vaccines are an alternative to injectable vaccines, because it is e.g. increasingly more difficult to incorporate new injectable vaccines into the existing paediatric vaccination programmes. In addition, mucosally delivered vaccines activate the mucosal immune system to elicit protective secretory IgA antibodies and cellular immunity. This is relevant for protection against viruses, bacteria and parasites that enter the body through mucosal surfaces and cause diseases of the intestinal, respiratory and genital tracts.

Oral administration of soluble protein antigens generally leads to systemic tolerance [1] but ingested particulate antigens and microbes in some instances generate mucosal and systemic immune responses [2] through stimulation of the gut-associated lymphoreticular tissue (GALT). Both soluble and particle-associated proteins given intranasally can, through uptake by the nasopharynx-associated lymphoretic-

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ular tissue (NALT), elicit local and distal mucosal as well as systemic immune responses [3]. Therefore, oral or nasal immunisation may also be useful against pathogens that enter the body through routes other than mucosa. Attenuated strains of bacterial pathogens such as *Salmonella typhi*, *Mycobacterium bovis* and *Bordetella pertussis* are being developed as vectors for mucosal immunisation, but suffer from the disadvantage that they tend to disseminate in the body [3] and can be unsafe in immuno-compromised persons.

Lactococcus lactis is ubiquitous in the human environment and in food. It is used for making cheese and buttermilk, and is generally recognized as safe (GRAS). *L. lactis* survives passage through the gastrointestinal tract but does not colonise the gut [4]. This lactic acid bacterium may therefore provide a safer alternative to attenuated pathogens for mucosal immunisation purposes [5]. Several bacterial or viral antigens have been expressed in *L. lactis* for use in oral and intranasal immunisations [6–13]. The advantage in relation to the potency of the elicited immune response of anchoring the antigens to the cell wall of the producing *L. lactis* cells as opposed to intracellular expression has been demonstrated by others [9,10,12–14]. Therefore, we focused in the present study on the cell wall presentation of the antigen.

In all previously described studies on the use of *L. lactis* in mucosal immunisation strategies, genetically modified vaccine strains were employed. Although *L. lactis* is an innocuous bacterium, its widespread use as a genetically modified strain in vaccinations, especially mucosal vaccinations, may cause undesirable shedding of recombinant DNA into the environment with the attendant risk of transfer to other organisms. In order to eliminate this risk we developed a non-genetically modified *L. lactis* support that allows highly efficient binding of proteins that are fused to a lactococcal peptidoglycan binding domain, the protein anchor (PA). For this purpose, *L. lactis* cells are pre-treated with an acid that removes surface components [15], leaving non-living particles that we termed Gram-positive enhancer matrix (GEM) [16,17]. The pre-treated and neutralized GEM particles are used to bind the antigen-PA fusion that was produced by another source (*in trans* binding). In this way, the antigen can be presented to the immune system as a bacterial particle that does not contain the recombinant DNA encoding the antigen.

We report here on the immunogenicity after oral delivery in rabbits of a *Plasmodium falciparum* parasite antigen presented in two different ways on the surface of *L. lactis*; (i) covalently anchored to the peptidoglycan layer of the cell wall of live genetically modified *L. lactis*, using an anchoring mechanism based on the LPXTG motif from, in this case, the lactococcal proteinase PrtP (cP) [18] and (ii) non-covalently rebound to non-genetically modified *L. lactis* GEM particles using the PA domain [15–17]. The *P. falciparum* protein used was the 45 kDa merozoite surface antigen MSA2 [19,20], antibodies against which have been associated with protection against the clinical symptoms of malaria [21] and, in some instances, inhibition of merozoite invasion of red blood cells in vitro [22].

2. Materials and methods

2.1. L. lactis strains and growth conditions

L. lactis-NZ9000 and NZ9700 were grown in M17 medium (Difco, MD, USA) containing 1% glucose at 30 °C in standing cultures. MSA2-recombinant *L. lactis* were grown in the same medium with 5 μg ml^{-1} of chloramphenicol for selection. MSA2-recombinant *L. lactis* were induced for expression of MSA2 variants by adding the culture supernatant of *L. lactis*-NZ9700, containing secreted nisin A, at a 1:1000 dilution and culturing overnight [23]. Overnight cultures of a strain of *L. lactis*-NZ9000 lacking the cell wall hydrolase AcmA (*L. lactis*-*ΔacmA*) [24] were used to prepare GEM particles as described before [17].

2.2. Cloning of MSA2 in L. lactis

The 819-nucleotide coding sequence of MSA2 [20] from the 3D7 isolate of *P. falciparum* cloned in pBluescript IISK+ [25], was used as source of *msa2*. The relevant *msa2* sequence was PCR amplified from this plasmid and then cloned into a pNZ8048-based lactococcal vector for the expression of MSA2 under the control of the nisin A inducible promoter P_{nisA} [23]. Plasmid pNG3041 (Fig. 1) expressed a 223-amino-acid (aa) fragment of MSA2 (beginning with the sequence KNES and ending with AATS, GenBank accession number A06129) lacking the MSA2 signal – and membrane-anchoring sequences. Instead it had the signal – and pro-sequence [nucleotides 1206–1766 in Ref. [18]] of the lactococcal cell wall-associated proteinase PrtP at the N-terminus for efficient secretion, together with a C-terminal anchor sequence (PA) derived from the *L. lactis* cell-wall hydrolase AcmA [corresponding to nucleotides 733–1488 in Ref. [24]]. The corresponding MSA2 fusion protein MSA2-PA is termed MSA2-nCov for reasons of clarity. MSA2-nCov is attached non-covalently to the cell wall of the producer cells through the protein anchor [15], and is also secreted into the culture medium after which it can be rebound non-covalently to lactococcal GEM particles [15–17]. Plasmid pNG3043 (Fig. 1) expressed the same 223-aa fragment of MSA2 as in pNG3041, with the signal and pro-sequence of PrtP at the N-terminus, but with a cell-wall spanning and covalent anchoring sequence of PrtP, cP, at its C-terminus [corresponding to nucleotides 6539–6914 in Ref. [18]]. This MSA2 fusion protein MSA2-cP is termed MSA2-Cov. The plasmids pNZ8048 (negative control), pNG3041 and pNG3043 were used to transform *L. lactis*-NZ9000, which has the *nisRK* genes in the chromosome needed for the nisin-induced activation of P_{nisA} [23], by electroporation [26].

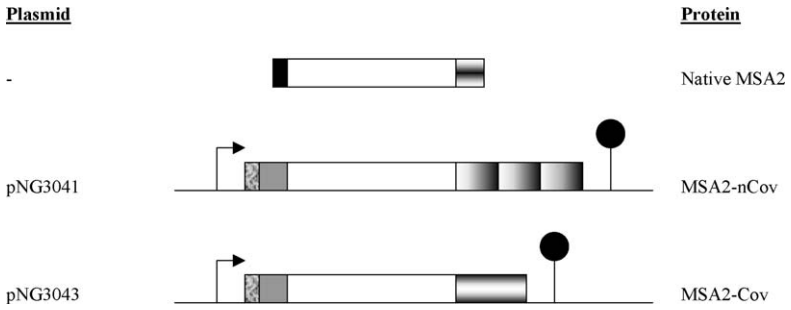

Fig. 1. Schematic drawings of the most relevant parts of pNG3041 and pNG3043. The indicated domains contain the nucleotide sequences encoding. Open box: MSA2 without signal sequence and GPI anchor sequence. Black box: native signal sequence of MSA2. GPI anchor sequence of MSA2. The arrow represents the nisin-inducible promoter of *L. lactis*. The lollypop represents a lactococcal transcriptional terminator sequence. signal sequence of PrtP. prosequence of PrtP. one repeat of the AcmA cell wall anchor. The three repeats constitute the entire cell-wall anchor domain (PA). cell wall anchor of PrtP (cP).

2.3. Quantification of MSA2 expression

Coomassie blue staining after SDS-PAGE was performed to quantitate the expression of MSA2 variants. Exponentially growing *L. lactis* cells were induced for 4 h with a supernatant containing nisin A and the cells were pelleted by centrifugation, washed once in distilled water and resuspended in buffer (20% sucrose, 10 mM Tris–HCl pH8.1, 10 mM EDTA and 50 mM NaCl) containing 500 $\mu g\,ml^{-1}$ lysosyme and 15 units ml^{-1} mutanolysin (Sigma, USA) and heated to 55 °C for 15 min. Then 1 volume of 2× concentrated Laemmli sample buffer was added and the sample was heated to 100 °C for 3 min. MSA2-nCov, non-covalently bound to *L. lactis* GEM particles, was extracted from GEMs with sample buffer at 100 °C. Ten microlitres of aliquots (5×10^8 cells) were analysed by SDS-PAGE (12% NuPage gels with MOPS buffer; Invitrogen, USA) and stained with Coomassie R250(G) stain. The intensity of the MSA2 bands was compared with BSA standards analysed similarly by SDS-PAGE.

2.4. Immunoblotting, immunofluorescence assay (IFA) and immuno-electron microscopy

Ten microlitres of aliquots of *L. lactis* extracts, prepared and separated by SDS-PAGE as described above, were transferred to Hybond blotting membranes and probed with a 1:10,000 dilution of a rabbit antiserum to MSA2 raised against an MSA2-GST fusion protein [25]. The blots were developed with 0.5 $\mu g\,ml^{-1}$ alkaline-phosphatase-labelled goat anti-rabbit IgG (H + L) antibodies followed by NBT/BCIP as a substrate.

For IFA, approximately 10^8 cells of pNG3043(MSA2-Cov), induced to express MSA2, or 10^8 *L. lactis* GEM particles with bound MSA2-nCov, were washed in PBS (0.1 M phosphate buffered saline, pH 7.2), and incubated with a 1:500 dilution of the rabbit anti-MSA2 serum in PBS with 2% BSA for 2 h at 28 °C. After washing with PBS, the samples were incubated with 10 $\mu g\,ml^{-1}$ Oregon green-conjugated goat anti-rabbit IgG antibodies (Molecular Probes, USA) diluted in PBS with 2% BSA for 1 h at 28 °C, and then viewed and photographed using a Zeiss microscope with incident UV illumination and the Zeiss Axiovision digital imaging system (Zeiss, Germany).

Immunogold electron microscopy was performed on whole mount preparations of glutaraldehyde-fixed MSA2 surface-displaying *L. lactis* cells and GEM particles on Formovar carbon-coated nickel grids. These were reacted sequentially with rabbit anti-MSA2 and Auroprobe 15 nm gold-labelled goat anti-rabbit IgG (Amersham, UK). Samples were then stained with 0.1% uranyl acetate and examined using a Philips CM10 transmission electron microscope at 100 kV (Philips, The Netherlands).

2.5. Vaccine preparations

Stocks of *L. lactis* and *L. lactis*-pNG3043 for immunisation were prepared from overnight cultures (the latter strain was induced overnight with nisin A for MSA2-Cov expression) and stored in aliquots of 10^{11} colony forming units (cfu) per ml growth medium containing 10% glycerol at −80 °C. The cells remain viable under these conditions and *L. lactis*-pNG3043 retains MSA2-Cov on its surface, as demonstrated by immunofluorescence assay (IFA).

Cultures of *L. lactis*-pNG3041 cells, induced for 4 h with nisin A to express MSA2-nCov, were centrifuged and the supernatant was collected and used as a source of secreted MSA2-nCov. *L. lactis* GEM particles were incubated with the cell-free supernatant containing MSA2-nCov at a concentration of 2×10^8 GEM particles per ml at 37 °C for 15 min with rotary shaking at 150 rpm. The GEM particles were then collected by centrifugation, washed twice in PBS and stored in PBS at −80 °C until further use at a concentration of 10^{11} particles per ml. *L. lactis* GEM particles retain MSA2-nCov on the surface under these conditions, as demonstrated by IFA.

2.6. Immunisations

New Zealand white rabbits obtained from the Medical Research Institute, Colombo, Sri Lanka were used for

Table 1
Serum antibody responses to immunisation through different routes with recombinant *L. lactis*-pNG3043 cells, which carry covalently bound MSA2-Cov

Route	Immunogen	0	1st	2nd	3rd	3-II	4th	Faecal Ab
i.m.	*L. lactis* MSA2-Cov	−ve	−ve	2	4(3)	3	5	−ve
Nasal	*L. lactis*	−ve	nd	nd	−ve	nd	nd	nd
Nasal	*L. lactis*	−ve	nd	nd	−ve	nd	nd	nd
Nasal	*L. lactis* MSA2-Cov	−ve	−ve	1	1(1)	1	1	nd
Nasal	*L. lactis* MSA2-Cov	−ve	−ve	−ve	1(1)	1	1	nd
Oral	*L. lactis*	−ve	nd	nd	−ve	nd	nd	−ve
Oral	*L. lactis*	−ve	nd	nd	−ve	nd	nd	−ve
Oral	*L. lactis* MSA2-Cov	−ve	−ve	1	2(1)	1	2	+ve
Oral	*L. lactis* MSA2-Cov	−ve	−ve	1	3(2)	1	3	+ve

Total serum antibody titres, determined by IFA at serial 10-fold dilutions, beginning at 1:10, after 0, 1, 2, 3 and 4 immunisations (0, 1st, 2nd, 3rd and 4th, respectively) are expressed as the negative logarithms to the base ten. Absence of a detectable IFA reaction at 1:10 dilution of serum is indicated as −ve. The numbers in parentheses in column 3rd are the corresponding titres of IgG antibodies determined by IFA with a specific anti-rabbit γ-chain antibody. 3-II is the serum collected 15 weeks after the third immunisation. If antibody was detected by IFA in the undiluted faecal extract this is reported as +ve. nd: not done; i.m.: intramuscular.

experimental immunisations in which the nasal and oral routes of administration were explored with the *L. lactis*-pNG3041(MSA2-Cov) vaccine. The care and use of animals were according to WHO guidelines (WHO/LAB/88.1). The rabbits were ear-bled 7 days prior to immunisation to obtain preimmune sera. Details of routes of immunisation and immunogens are given in Table 1. Each group consisted of two rabbits. As a positive control, *L. lactis*-pNG3041(MSA2-Cov) was given intramuscularly (i.m.) to one rabbit. Prior to immunisations, stocks of *L. lactis* (no MSA2 expression, negative control) and *L. lactis*-pNG3041(MSA2-Cov) were thawed, washed and resuspended in buffer at the appropriate concentration for immunisations. Intramuscular injections were performed with a total of 5×10^9 cfu in 100 μl PBS into both rear hind legs. Nasal immunisation involved delivering a total of 10^{10} cfu in 100 μl PBS into the two nostrils with a pipette. Prior to oral immunisation, the rabbits were deprived of water and food for 6 h. They were then each fed 5×10^{10} cfu resuspended in 1 ml of 0.2N $NaHCO_3$, 0.5% glucose and 5% casein hydrolysate. This dose was repeated for three successive days to obtain reproducible oral immunisation. All immunisations were repeated twice at 3 weeks intervals. Blood was collected 2 weeks after each immunisation to prepare sera termed bleeds 1st, 2nd and 3rd, respectively. After three immunisations the rabbits were rested for 15 weeks. Serum was then collected (termed bleed 3-II) to test the decrease in antibody titres. The rabbits were given a fourth and final immunisation 16 weeks after the third immunisation, and serum collected 2 weeks later (termed 4th), to confirm boosting of immunity. The fourth oral immunisation, however, consisted of two, and not three, successive days of feeding 5×10^{10} cfu. Collected sera were stored at −20 °C until use.

New Zealand white rabbits obtained from Harlan laboratories, The Netherlands, were used for immunisations in which the *L. lactis* GEM particles carrying MSA2-nCov and the *L. lactis*-pNG3041(MSA2-Cov) vaccines were compared. The care and use of animals were according to institutional guidelines. The rabbits were ear-bled prior to immunisation to obtain preimmune sera. Details of the immunisation and immunogens are given in Table 2. Each group consisted of two rabbits. Prior to immunisations, stocks of *L. lactis*-pNG3041(MSA2-Cov) and *L. lactis* GEM particles with bound MSA2-nCov were thawed, washed and resuspended in 20% sucrose for oral immunisations. Prior to oral immunisation, the rabbits were deprived of water and food for 2–4 h. They were then each fed using a plastic disposable pipette tip with 1 ml containing 5×10^{10} cfu *L. lactis*-pNG3041(MSA2-Cov) or 5×10^{10} GEM particles with bound MSA2-nCov. Each dose was repeated for three successive days to obtain reproducible oral immunisation. Altogether, three oral immunisations were given at 3 weeks intervals. Rabbits were ear-bled 2 weeks after each immunisation to obtain sera for antibody assays. The sera were stored at −20 °C until use.

All vaccines were administered without additional adjuvants. Adverse effects consequent to the immunisations were not observed indicating that live recombinant *L. lactis* as well as non-living *L. lactis* GEM particles were well tolerated by the animals.

Table 2
Serum antibody responses after oral immunisation with recombinant *L. lactis*-pNG3043 carrying covalently bound MSA2-Cov or *L. lactis* GEM particles containing non-covalently bound MSA-nCov

Immunogen	0	2nd	3rd	3rd IgG
L. lactis MSA2-Cov	−ve	3	4	4
L. lactis MSA2-Cov	−ve	2	4	4
L. lactis GEM MSA2-nCov	−ve	2	4	4
L. lactis GEM MSA2-nCov	−ve	2	4	4

Titres determined by IFA at serial 10-fold dilutions, beginning at 1:10, are expressed as the negative logarithms to the base ten. No detectable reaction at a 1:10 dilution of the serum is shown as −ve; 0, 2nd and 3rd refer to preimmune serum and serum obtained after 2 and 3 immunisations, respectively; 3rd IgG is the IgG antibody titre in the serum after three immunisations, as determined with a rabbit γ chain-specific secondary antibody.

2.7. Detection and measurement of serum antibody response

The detection of typical bunch of grape-like patterns of fluorescence on segmented schizonts of 3D7 *P. falciparum* is based on antibody recognition of near-native MSA2 on the surface of the malaria parasite [19]. This method was used to determine the MSA2 antibody titre in the sera of immunised rabbits. IFA was performed on acetone–methanol fixed late-stage 3D7 parasites as previously described [19]. Ten-fold serial dilutions of the antisera in 2% BSA were used in IFA to determine the titre. The greatest dilution of serum giving a discernible bunch of grapes pattern on late schizonts was taken as the titre. For detection of all antibody isotypes, FITC-conjugated sheep anti-rabbit Ig (Silenius, Australia) or Oregon Green-conjugated goat anti-rabbit Ig with H + L specificity (Molecular Probes, USA), was used as secondary antibody. For detection of IgG antibodies only, a FITC-conjugated mouse monoclonal antibody RG-96, with specificity against rabbit γ chains (Sigma, USA), or a fluorescein-conjugated, affinity-purified, rabbit antibody with specificity against rabbit γ chains (Rockland, USA), was used instead.

A total cell extract of *L. lactis* NZ9000 was used as target antigen for immunoblotting with the rabbit sera to determine the anti-carrier response.

2.8. Faecal antibody responses

Fresh faecal pellets were collected from the rabbits 2 weeks after the third immunisation and stored at −20 °C until required. One millilitre of PBS containing 1% BSA and 1 mM phenylmethyl suphonyl fluoride was then added per 100 mg of faeces and incubated overnight at 4 °C. The sample was vortexed to disrupt all solid material and centrifuged at 16,000 × *g* for 5 min. The supernatant containing extracted antibody was stored at −20 °C until testing by IFA. FITC-conjugated sheep anti-rabbit Ig (Silenius, Australia) was used as the secondary antibody.

3. Results

3.1. Expression of MSA2

The expression formats of MSA2 are schematically drawn in Fig. 1. Expression of the MSA2 fusion proteins was analysed in Coomassie-stained gels and in Western blots. An example of such gels and blots is given in Fig. 2. MSA2-Cov was present as a 70-kilo Dalton (kDa) protein in the total cell extract of *L. lactis*-pNG3043 (Fig. 2, lane 2). MSA2-nCov was secreted by *L. lactis*-pNG3041 as a 65 kDa protein (Fig. 2, lane 4). The secreted MSA2-nCov was bound to *L. lactis* GEM particles (Fig. 2, lane 6). By comparative protein gel electrophoresis experiments using BSA protein standards we estimated that *L. lactis* produced approximately 800 ng MSA2-Cov per 5×10^8 cells. The same number of GEM particles contained about 600 ng of MSA2-nCov (data not shown). This corresponds to approximately 1.4×10^4 MSA2-Cov molecules per cell for *L. lactis*-pNG3043 and about 10^4 MSA2-nCov molecules per GEM particle.

3.2. Location of MSA2 on L. lactis cells and GEM particles

The surface localization of MSA2-Cov on *L. lactis*-pNG3043 cells and of MSA2-nCov on GEM particles was confirmed by immunofluorescence with rabbit anti-MSA2 serum (Fig. 3) and by immuno-electron microscopy (Fig. 4). The microscopic observations suggest that surface-exposed MSA2-Cov is mainly found in the region of the newly forming septum in dividing cells and at the poles of the *L. lactis*-pNG3043 cells. MSA2-nCov was more evenly distributed on

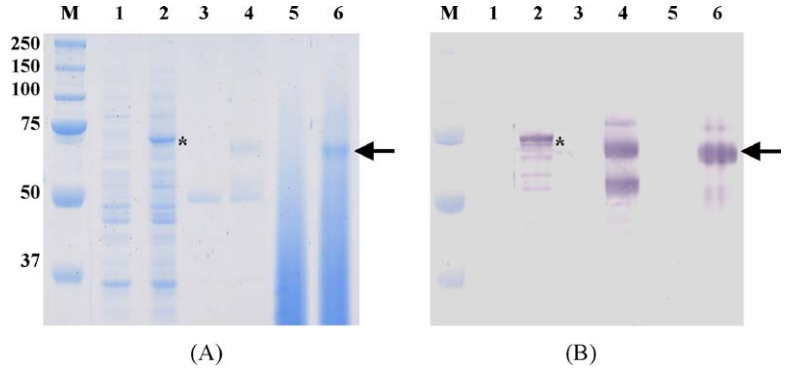

Fig. 2. Panel A: SDS-PAGE analysis of Coomassie-stained proteins in cell lysates (5×10^8 cells) prepared from—lane 1: *L. lactis*-pNZ8048, empty cloning vector; lane 2: *L. lactis*-pNG3043(MSA2-Cov); lane 3: the equivalent of 1 ml culture supernatant of *L. lactis*-pNZ8048; lane 4: the equivalent of 1 ml culture supernatant of *L. lactis*-pNG3041(MSA2-nCov); lane 5: *L. lactis* GEM particles; lane 6: *L. lactis* GEM particles with MSA2-nCov absorbed from 1.25 ml *L. lactis*-pNG3041(MSA2-nCov) cell-free culture supernatant. M: molecular weight marker (precision Plus Prestained Marker, BioRad), the sizes of which are indicated in kDa in the left margin. The asterisk and the arrow indicate the positions of MSA2-Cov and MSA2-nCov, respectively. Panel B: Immunoblot decorated with anti MSA2-GST antibodies to detect MSA2-containing fusion proteins. Content of the lanes is the same as in panel A.

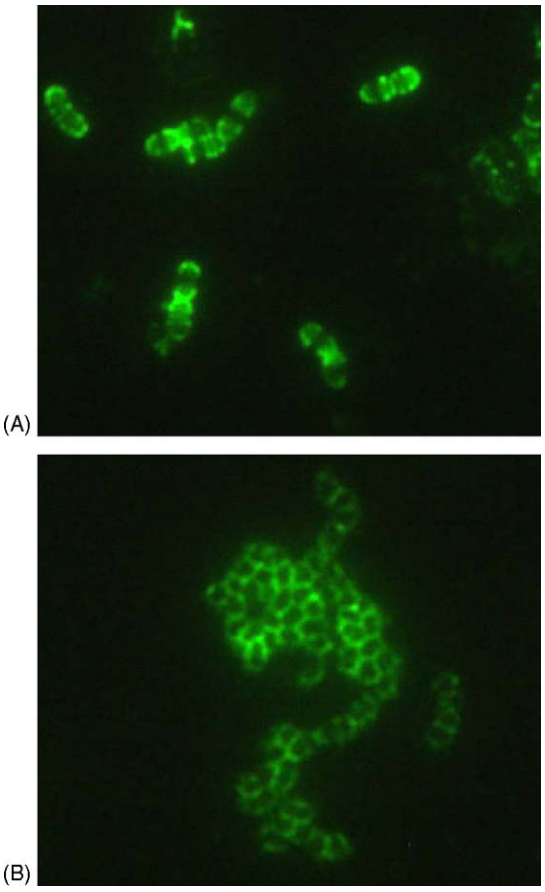

Fig. 3. Immunofluorescence assay (IFA) showing reaction of rabbit anti-MSA2 antibodies on: (A) *L. lactis*-pNG3043 induced for 4 h with nisin to express MSA2-Cov; (B) *L. lactis* GEM particles after absorption with MSA2-nCov from the cell-free culture supernatant of *L. lactis*-pNG3041 induced for 4 h to express MSA2-nCov. The negative controls did not show any fluorescence (data not shown).

L. lactis GEM particles (Figs. 3 and 4). In any case, similar levels of MSA2 seem to be surface displayed by these two presentation formats.

The most efficient mucosal route for the delivery of MSA2 was first determined using the live recombinant *L. lactis* antigen surface display system, i.e. *L. lactis*-pNG3043(MSA2-Cov).

3.3. Immunogenicity of MSA2-Cov delivered by mucosal routes

New Zealand white rabbits were used to examine the oral and nasal routes of immunization. The immunisations, including two boosters, with recombinant *L. lactis*-pNG3043(MSA2-Cov) delivered, per rabbit, a total of 48 and 720 μg of MSA2-Cov protein after nasal and oral immunisations, respectively. One rabbit obtained three intramuscular (i.m.) immunisations with *L. lactis*(MSA2-Cov) as a positive control (totally 24 μg MSA2-Cov). Immunofluorescence reactivity against the surface of *P. falciparum* merozoites was used to assay antibodies in sera and faecal extracts, as this measures antibodies reacting with near-native MSA2 protein (see Section 2). The IFA results of the immunised rabbits are summarised in Table 1. A single immunisation with *L. lactis*(MSA2-Cov) administered i.m., nasally or orally did not yield anti-MSA2 serum antibodies detectable by IFA. A further boosting immunisation was needed in all cases to detect antibodies. It is apparent that the positive control, i.m. immunisation with *L. lactis*(MSA2-Cov), led to good serum antibody titres. The titres waned 15 weeks after the third injection but could be boosted to the original levels by a fourth i.m. injection. Levels of IgG antibodies after three immunisations, as measured with an FITC-conjugated mouse monoclonal antibody against rabbit γ chains, were one order of magnitude less but still indicate that IgG antibodies constitute the major portion of the antibody response against

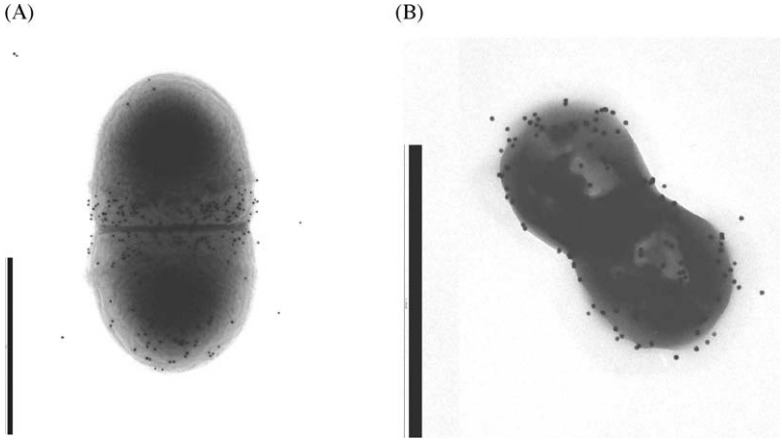

Fig. 4. Electron micrographs showing, by immunogold labelling using rabbit anti-MSA2 antiserum and gold-labelled goat anti-rabbit IgG, MSA2 on the surface of: (A) *L. lactis*(MSA2-Cov), 20 min after induction with nisin A; (B) *L. lactis* GEM particles with attached MSA2-nCov. The black bars indicate a size of 1 μm. For detection, secondary antibodies labelled with 15 nm gold particles were used, both in (A) and (B). Because of the used whole mount sample preparation procedure (see Section 2), the GEM particles shrink and the photograph was more enlarged in order to obtain similar sized bacterial particles.

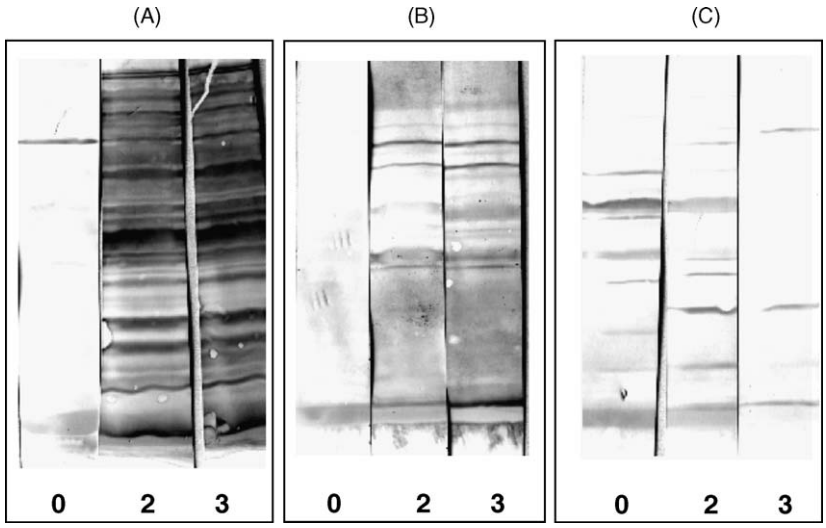

Fig. 5. Immunoblots showing reaction of sera of immunised rabbits with total protein extracts of 5×10^8 *L. lactis* cells per lane—lane 0: preimmune serum; lane 2: serum after the 2nd immunisation; lane 3: serum after the 3rd immunisation. Serum dilution 1:100. Panel A: Serum of a rabbit immunised i.m. with *L. lactis*(MSA2-Cov). Panel B: Serum of a rabbit orally immunised with *L. lactis*(MSA2-Cov). Panel C: Serum of a rabbit orally immunised with *L. lactis* GEM particles with bound MSA2-nCov.

MSA2-Cov expressed on cells of *L. lactis*-pNG3043(MSA2-Cov) after i.m. immunisation.

Intranasal immunisation with *L. lactis*(MSA2-Cov) elicited low titres (10^{-1}) of antibodies after three immunisations. The oral immunisation procedure led to higher antibody titres (10^{-2} to 10^{-3}) than did the intranasal immunisation. Antibody levels decreased significantly by 15 weeks but could be boosted to original levels by a subsequent (4th) oral immunisation. The results with the anti-rabbit γ-chain antibody and the boosting suggest that much of the antibody present in the serum after oral immunisation is IgG.

MSA2-specific antibodies were detectable in undiluted extracts of faecal pellets after oral immunisation, but not after i.m. immunisation with *L. lactis*(MSA2-Cov).

3.4. Immunogenicity of MSA2-Cov and MSA2-nCov after oral immunisation

Based on the results obtained for MSA2-Cov, we concluded that the preferred route for mucosal delivery in rabbits of the MSA2 antigen by live recombinant *L. lactis* is oral administration. We next determined the capability of non-living non-genetically modified lactococcal GEM particles carrying non-covalently bound MSA2-nCov to elicit systemic MSA2-specific antibodies after oral administration. A comparison was made with *L. lactis*(MSA2-Cov). *L. lactis*(MSA2-Cov) delivered a total of 720 μg MSA2-Cov while GEM particles delivered 540 μg of MSA2-nCov after three immunisations. The rabbit anti-MSA2 serum antibody responses to the immunisations were characterised by IFA and are summarised in Table 2. The results show that specific antibodies against near-native MSA2 were detectable after two immunisations (titres of 10^{-2} to 10^{-3}), and that antibody titres increased after a third immunisation (10^{-4}), in all instances. The antibodies were predominantly of the IgG subclass in all cases.

3.5. Anti-carrier response

The immune response to the carrier may be of influence on the safety of the delivery system in humans. Also the delivery route may be of influence on the anti-carrier response. As a first exploration of this issue we examined the anti-carrier antibody response in more detail in immunoblots in which a total lysate of *L. lactis* cells was used as a target for serum from rabbits immunised with the different MSA2 presentation formats (Fig. 5). The staining bands in the lanes indicate that *L. lactis* proteins present at that position in the SDS-PAA gel reacted with the indicated rabbit antiserum. A pronounced anti-*L. lactis* protein response was observed after 2 or 3 i.m. administrations of the live recombinant *L. lactis* vaccine (Fig. 5A). This response was clearly reduced after oral delivery of the vaccine (Fig. 5B). The anti-*L. lactis* response was even more minimized in the rabbit orally immunised with the GEM particle vaccine (Fig. 5C). It is clear from these results that mucosal delivery reduces the anti-carrier response and that the *L. lactis* GEM particles generate less anti-carrier antibody responses than the live recombinant *L. lactis* cells.

4. Discussion

We investigated the immunogenicity of the putative protective MSA2 antigen of *P. falciparum* merozoites in mucosal vaccines. It was also our aim to evaluate the feasibility of a

novel bacterial antigen display system that avoids the use of recombinant DNA in the vaccine. For this purpose, we compared MSA2 vaccines based on a live recombinant *L. lactis* surface display system that exploits the commonly used covalent LPXTG-type anchoring cassette [9,10,12–14,27,28], with a non-living non-genetically modified *L. lactis* surface display system, the GEM particles. The GEM particles can be loaded with the antigen when the latter is fused to the non-covalent cell wall binding domain (PA) of the lactococcal protein AcmA. Others have already demonstrated that non-living recombinant lactococci are as efficient as live *L. lactis* cells in eliciting antibody responses in mucosal immunizations [7]. The AcmA cell wall binding domain PA has been used to display antigens on live recombinant [27,28] or non-recombinant live *L. lactis* cells [29]. Here we used acid pre-treated *L. lactis* cells, which we termed GEM particles [16], for antigen delivery because this pre-treatment enhances the binding capacity for PA fusion proteins [15–17]. Moreover, the use of GEM particles in vaccines prevents the dissemination of recombinant DNA in the environment. The results of the oral immunisations confirm that non-living *L. lactis* GEM particles are equally efficient in eliciting antigen-specific antibody responses as living *L. lactis* cells. Consequently, the present results show that *L. lactis* GEM particles, which consist of peptidoglycan largely devoid of lipotechoic acid and lacking DNA [16], known immunostimulants [30,31], can also efficiently stimulate antigen presenting cells without the use of additional adjuvants. In addition, GEM particles elicit less anti-carrier response than live *L. lactis* cells. Not requiring additional adjuvant and a minimal anti-carrier response would be desirable characteristics for any vaccine to be used in human vaccinations. Further investigations are warranted to reveal the mechanism of immune stimulation.

Our results with the recombinant *L. lactis* delivery system suggest that oral immunisation leads to higher levels of serum antibodies than the corresponding nasal immunisation procedure employed. A similar observation was made by Pei et al. [13] in immunisations with *L. lactis* expressing the SARS-coronavirus nucleocapsid protein. Also other investigators observed significant systemic immune responses after oral immunisations with recombinant lactococci [7–10] and in some cases protection against a lethal challenge was obtained [7,10,11]. Protection against infection with *P. falciparum* can not be evaluated in a challenge model using non-primate animals. The anti-MSA2 serum antibodies generated in the present study in the oral immunisations with both delivery formats, react with near-native antigen on merozoites, as evidenced by the IFA studies on intact merozoites. Reaction of the antibodies with merozoite surfaces is related to their biological efficacy, i.e. the inhibition of red cell invasion. In addition, the demonstration of systemic IgG antibodies whose levels can be boosted implies that MSA2-specific T_h cells are activated through mucosal immunisation. The detection of MSA2-specific IgA antibodies in the extracts from faecal pellets of rabbits immunised orally is consistent with the observation of others [7,10,11,32,33]. Therefore, the findings suggest that foreign proteins expressed in *L. lactis* can also be used in oral vaccination procedures to elicit protective secretory antibodies in the gut.

Antibody titres achieved by using both *L. lactis* display formats of MSA2 are better than those achieved by immunisation with plasmid DNA [34] or peptide-diphtheria toxoid conjugates [35] through the intramuscular and intradermal routes. Intramuscular immunisation with synthetic peptide polymers based on MSA2 B-cell epitopes elicited high-titre antibodies against the immunising peptide, which reacted only poorly with near-native MSA2 on merozoites [36]. While very high titres (10^{-6}) of antibodies reacting with near-native MSA2 is achievable in animals with recombinant MSA2 in intramuscular immunisations, this required the use of Freund's adjuvant, which is not acceptable for human use [25]. Adjuvants with fewer side effects that have been tried on humans with recombinant MSA2, have yielded poorer antibody titres [37]. Therefore oral immunisation with *L. lactis* MSA2 display formats is a promising method of generating systemic anti-MSA2 antibodies in man for protection against malaria.

The use of heterologous proteins anchored through the PA to non-genetically modified *L. lactis* GEM particles is as effective as the use of living recombinant *L. lactis* cells for eliciting systemic IgG antibodies against the protein of interest. The apparent advantages of the GEM approach are: (i) it avoids the need for introducing recombinant cells or additional adjuvants into vaccine recipients; (ii) it elicits a weaker immune response against the vector, thereby increasing safety and possibly permitting a greater number of immunisations than feasible with intact *L. lactis*; (iii) the process more easily permits immunisation with different combinations of antigens on the same particle [16]. Experiments are presently in progress to determine whether proteins absorbed on the GEM particles are able to induce antigen-specific systemic Tc cells, which could further widen the use of the GEM particles in vaccine preparations.

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