

Oral vaccination of mice against rodent malaria with recombinant *Lactococcus lactis* expressing MSP-1₁₉

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Abstract

AIM: To construct the recombinant *Lactococcus lactis* as oral delivery vaccination against malaria.

METHODS: The C-terminal 19-ku fragments of MSP1 (MSP-1₁₉) of *Plasmodium yoelii* 265-BY was expressed in *L. lactis* and the recombinant *L. lactis* was administered orally to BALB/c and C57BL/6 mice. After seven interval vaccinations within 4 wk, the mice were challenged with *P. yoelii* 265-BY parasites of erythrocytic stage. The protective efficacy of recombinant *L. lactis* was evaluated.

RESULTS: The peak parasitemias in average for the experiment groups of BALB/c and C57BL/6 mice were 0.8±0.4% and 20.8±26.5%, respectively, and those of their control groups were 12.0±0.8% and 60.8±9.6%, respectively. None of the BALB/c mice in both experimental group and control group died during the experiment. However, all the C57BL/6 mice in the control group died within 23 d and all the vaccinated mice survived well.

CONCLUSION: The results imply the potential of recombinant *L. lactis* as oral delivery vaccination against malaria.

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Key words: *Lactococcus lactis*; Oral delivery vaccination; Malaria

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INTRODUCTION

The development of efficacious vaccines against malaria is one of the greatest challenges for the application of current life sciences in infectious diseases. The easiness of administration of a vaccine provides an attractive alternative to continue drug treatments in a population exceeding hundreds of millions of people with limited health care resources. Merozoite surface protein 1 (MSP1) is present in all species of *Plasmodium*^[1,2], and has been widely studied as the major candidate for vaccine against malaria^[3-5]. The high level expression of MSP1 by *Plasmodium* in the asexual stage is closely related to its invasion into erythrocytes. MSP1 can be proteolytically cleaved into five fragments by two processing steps after the maturation of merozoite, with the carboxyl-terminal 19-ku fragment (MSP-1₁₉) remaining on the merozoite surface^[6-8]. The MSP-1₁₉ comprises two epidermal growth factor (EGF)-like modules. Antibodies directed to this fragment have been shown to inhibit the invasion of *Plasmodium falciparum* into erythrocyte *in vitro*^[9,10] and intranasal or subcutaneous immunization may protect mice against the challenge of *Plasmodium yoelii* asexual blood-stage parasite^[11]. The recombinant MSP-1₁₉ has been expressed in several host organisms, such as *Escherichia coli*^[12], *Saccharomyces cerevisiae*^[11], *Bacillus Calmette-Guerin* (BCG)^[13], and Baculovirus^[14]. It has also been proven to be immuno-effective against the challenge of parasite. In vaccination experiments with recombinant MSP-1₁₉ from *P. yoelii*, immunized mice were protected against challenge with blood-stage parasites, and the protection was confirmed to be largely mediated by antibodies^[15-17].

The development of efficient mucosal vaccines is one of the hotspots in modern vaccinology. One approach to deliver the protective antigens to the mucosal surfaces is to use live bacteria carrying plasmids responsible for the expression of specific antigen. Until recently most of these are derived from attenuated pathogenic microorganisms, such as *Salmonella typhi*^[18] and *Chlorella*^[19]. As an alternative to this strategy, non-pathogenic food grade bacteria such as lactic acid bacteria are being focused for their efficacy as live antigen carriers^[20]. *Lactococcus lactis* has a long history of being used in food fermentations and has been, therefore, generally regarded as a safe (GRAS) status^[21]. This food-grade lactic acid bacterium is able to survive through the gastrointestinal tract of human beings and other animals, with a retention time of 2-3 d, but it does not invade or colonize the mucous and does not evoke strong host im-

immune responses^[21]. The availability of various food-grade genetic engineering systems for *L. lactis*^[22] makes the bacteria a potentially functional food or medicines by expressing heterogeneous peptides. Recent report using *L. lactis* preloaded with a bacterial antigen, tetanus toxin fragment C of *Clostridium tetanus*, demonstrated the feasibility of this approach: a protective systemic antibody response was elicited after nasal or oral immunization of mice^[21]. Similar study was carried out by Lee *et al*^[23], in which urease subunit B (UreB) gene of *Helicobacter pylori* was expressed in *L. lactis* MG1363 and the recombinant bacterium was used as an oral vaccine against *H. pylori* infection in mice. However, in this case no protective effect was observed, which implied that the adjuvant effects of *L. lactis* are likely to be insufficient to produce an effective immune response to protect against *H. pylori* challenge, when used to deliver a weak immunogen like UreB.

Since the use of oral (or other mucosal) routes for immunization against malaria is also desirable due to the easiness of administration, we attempted *L. lactis* as the live vehicle for vaccine development against malaria. In this work, we showed that the oral immunization, with recombinant *L. lactis* constitutively expressing MSP-1₁₉ antigen, could protect BALB/c and C57BL/6 mice against malaria parasites challenge.

MATERIALS AND METHODS

Genes, plasmids, bacteria, and malarial parasites

The DNA fragment encoding for MSP-1₁₉ domain was amplified from the genomic DNA of *P. yoelii*. Plasmid pTRKL2 was from Prof. Todd R. Klaenhammer at Food Science Center, North California University, USA^[24]. Cloning vector pBluescriptSKII(+) was from Stratagene (La Jolla, CA, USA), and fusion-protein expression vector pGEX-5X-3 was from Amersham Pharmacia. *L. lactis* LM2345 was from Prof. Keith Thompson at Agriculture and Food Science Center, Newforge Lane, Northern Ireland. *Lactobacillus brevis* (ATCC8287) and *Bacillus subtilis* BR151 (ATCC33677) were purchased from the American Type Culture Collection. *P. yoelii* 265-BY was by Professor Weibin Guan at Second Military Medical University, Shanghai, China.

Construction of expression vector

The plasmid for the expression of MSP-1₁₉ fragment was constructed by conventional DNA recombination manipulation. The promoter region and the first five amino acids of the signal peptide-coding region of S-layer protein A (*SlpA*) gene (nucleotides 1-282, GenBank Z14250) was amplified by polymerase chain reaction (PCR) from genomic DNA of *L. brevis* with forward primer GCTGAGCTCGATTACAAAGGCTTTAAGCAGGT-TAGTGAC (with *SacI* site) and reverse primer GTCG-GATCCTAAACTTGATTGCATAATCTTCTTCCTCC (with *BamHI* site). The DNA fragment encoding MSP-1₁₉ (nucleotides 5 040-5 451, GenBank AF165928) was amplified from the genomic DNA of *P. yoelii* 265BY, with

forward primer ACGGGATCCAA CACATAGCCT-CAATAGCT (with *BamHI* site) and reverse primer ACGGAATTCTAGCTGG AAGAACTACAGAA (with *EcoRI* site). The terminator of N-acetylmuramoyl-L-alanine amidase (*cmfB*) (nucleotides 2 187-2 471, GenBank M81324) was amplified from *Bacillus subtilis* BR151 by PCR with forward primer CTCGAGCTCCACAAGC-TATTCATGAC (with *XhoI* site) and reverse primer GG-TACCTCTCT GCACTCACTG ACACA (with *KpnI* site). The PCR products were cloned on T-vector (Promega, USA) first, and then joined together in a tandem way on pBluescriptSKII(+) with restriction enzyme pairs of *SacI*/*BamHI*, *BamHI*/*EcoRI*, and *XhoI*/*KpnI* respectively to obtain plasmid pSK-PSGT. For expression of MSP-1₁₉ in *L. lactis*, pSK-PSGT was then digested with *PvuII* to release the 1.6-kb blunt-end fragment, which was then inserted into the *EcoRV* site of shuttle vector pTRKL2^[24]. The final construct was referred as pL2-PSGT. For expression of fusion protein GST-MSP-1₁₉ in *E. coli*, the DNA fragment encoding MSP-1₁₉ was derived by PCR and joined to fusion protein expression vector pGEX-5X-3 with *BamHI* and *EcoRI* sites. The derived plasmid was noted as pGEX-MSP-1₁₉.

Transformation

Electroporation^[25] was used to transform *L. lactis* LM2345 with pL2-PSGT. CaCl₂ method was used to transform *E. coli* BL21 with plasmid pGEX-5X-3.

Preparation of antiserum against *P. yoelii* 265-BY

The antiserum against *P. yoelii* 265-BY was prepared from BALB/c mice infected with 10⁴ asexual blood stage parasites. The serum was collected from the eye veins 4 d after burst with 10⁴ parasites one month after infection, and stored at 4 °C.

Expression and analysis of MSP-1₁₉ in *E. coli* and *L. lactis*

The BL21 transformant harboring plasmid pGEX-MSP-1₁₉ was cultured in L-broth to A₆₀₀ around 1.0, and was treated with supplement containing 1.0 mmol/L of isopropylthio-β-galactoside (IPTG) for 3 h. The fusion protein GST-MSP-1₁₉ was purified from cell lysate by affinity chromatography of Glutathione Sepharose-4B according to manual instruction provided by the manufacturer. The purified fusion protein was used as positive control of immunoblotting. To express MSP-1₁₉ in *L. lactis*, the transformant harboring plasmid pL2-PSGT was cultured overnight in MRS medium^[26] supplemented with 10 mg/L erythromycin. The cells were collected by centrifuge, and the lysates were analyzed by using SDS-polyacrylamide gel electrophoresis analysis followed with immunoblotting by using antiserum against *P. yoelii* 265-BY (1:500 dilution). All other operations were performed following standard protocols^[27].

Oral immunization of BALB/c and C57BL/6 mice with recombinant *L. lactis*

Oral immunization experiments were performed according

to the protocol described by Robinson *et al.*^[21]. The BALB/c and C57BL/6 mice were divided into groups of 10 mice and fed for 6–8 wk. The test group was administered with recombinant *L. lactis* constitutively expressing MSP-1₁₉, and the control groups were administered with free *L. lactis* bacteria or phosphate-buffered saline (PBS). For BALB/c mice, every dose containing 5×10^9 bacterial cells in the suspension buffer (0.2 mol/L sodium bicarbonate, 5% Casino acids, and 0.5% glucose); and for C57BL/6 mice (two dosage groups), every dose containing 5×10^9 or 1×10^8 cells were administered. All the groups were administered with recombinant *L. lactis* on d 1, 2, 3, 29, 30, 31, and 36, respectively.

Challenge infections and evaluation of protective efficacy
Mice in each group were challenged on d 49 with 1×10^5 asexual blood stage *P. yoelii* parasites obtained from a donor mouse. Parasitemia was monitored every two days after challenge using microscopic examination of blood film with Giemsa staining.

RESULTS

Expression of fusion protein GST-MSP-1₁₉ in *E. coli*

The fusion protein GST-MSP-1₁₉ was expressed in *E. coli* BL21 transformed with plasmid pGEX-MSP-1₁₉. The transformed BL21 cells were cultured in L-broth until the A_{600} reached 1.0, and then induced with 1.0 mmol/L IPTG for 3 h. Total proteins of BL 21 *E. coli* cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in lane 4 of Figure 1, the thick band of the expressed protein showed a molecular weight of 45 ku, matching well with the theoretical value of fusion protein GST-MSP-1₁₉. The expressed fusion protein was about 40% of the total protein of *E. coli* cells. Most of the fusion protein was found in inclusion body, but a small fraction was soluble. Glutathione Sepharose-4B affinity chromatography was carried out with the soluble fraction of *E. coli* cell lysate, and the derived fusion protein

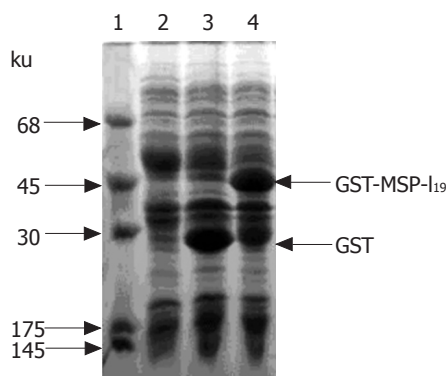


Figure 1 Expression of fusion protein in *E. coli* BL21 cells. Coomassie brilliant blue-stained 12% SDS-polyacrylamide gel. lane 1, protein markers; lane 2, total protein of BL21 cells; lane 3, total protein of *E. coli* BL21 transformed with plasmid pGEX-5X-3 with IPTG induction; lane 4, total protein of *E. coli* BL21 cells harboring plasmid pGEX-MSP-1₁₉ after with IPTG induction. The arrows indicate the positions of GST and fusion protein GST-MSP-1₁₉.

was used as the positive control of immunoblotting.

Expression of MSP-1₁₉ in *L. lactis*

For the expression of MSP-1₁₉ in *L. lactis*, the transformant harboring plasmid pL2-PSGT was cultured overnight at 30 °C in MRS medium^[26] supplemented with 10 mg/L erythromycin. The cells were collected by centrifuge, and the lysates were analyzed with SDS-PAGE. However, no obvious protein bands around 19 ku were detected. Immunoblotting with antiserum against *P. yoelii* 265-BY was applied to check if there was low-level expression of MSP-1₁₉. As a result, the presence of MSP-1₁₉ was confirmed by immunoblotting as shown in lane 3 of Figure 2. The stained protein band at 19 ku was a little bit broad. This might be the result of partial degradation of MSP-1₁₉ in *E. coli* cells. At least part of the expressed MSP-1₁₉ was in its native structure, since the antiserum prepared by *P. yoelii* parasites infection is considered to preferentially recognize the MSP-1₁₉ fragment located on erythrocyte membrane with the native conformation.

In lane 2 of the positive control, two protein bands were stained: one was at 45 ku, and the other was slightly below 45 ku, but not detectable on SDS-PAGE with Coomassie brilliant blue staining. When fusion protein is isolated from inclusion body of corresponding *E. coli* and refolded by rapid dilution method, only one protein band could be stained (data not shown). Therefore, we concluded that the low molecular weight protein was the degraded fusion protein present in the soluble fraction of *E. coli*.

Evaluation of protective immunity induced by recombinant *L. lactis*

Previous work of Tian *et al.*^[28] indicated that mice with different genetic backgrounds may have quite different responses to *P. yoelii* infection. C57BL/6 mice showed the highest level against challenge with infected erythrocytes after immunization with recombinant proteins consisting of the PyMSP-1 C terminus in adjuvants. In this work, two strains of mice, BALB/c and C57BL/6, were used for oral

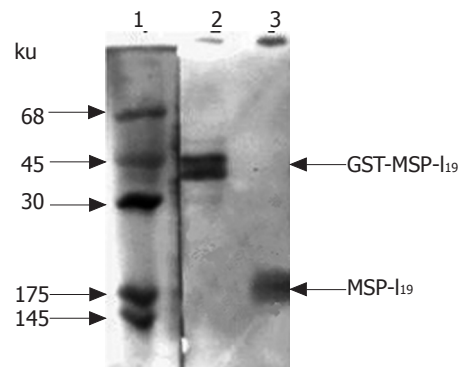


Figure 2 Immunoblotting analysis of MSP-1₁₉ expressed in *L. lactis*. Protein samples were first analyzed on 12% SDS-polyacrylamide gel and then transferred on nitrocellulose membrane followed by immunostaining with antiserum prepared by infecting mouse with *P. yoelii* parasites. lane 1, protein markers stained by amido black; lane 2, positive control of fusion protein GST-MSP-1₁₉ purified from *E. coli* cell lysate expressing the fusion protein by pGEX-MSP-1₁₉; lane 3, total protein of *L. lactis* cells harboring plasmid pL2-PSGT. The arrows indicate the position of fusion protein GST-MSP-1₁₉ and MSP-1₁₉.

immunization for comparison.

For BALB/c mice, two control groups were designed. Mice were administered with phosphate-buffered saline in control group 1, with 5×10^9 per dose of original *L. lactis* cells in control group 2, and with 5×10^9 per dose of *L. lactis* cells carrying pL2-PSGT construct in the test group, respectively. After seven doses of vaccination, each mouse was challenged with 1×10^5 asexual blood stage parasites. The parasitemias were measured from the next day of parasite challenging. The average and standard deviation of each group are shown in Figure 3. The peak p-parasitemias were $0.8 \pm 0.4\%$ at d 4 for test group, $16.0 \pm 1.2\%$ at d 8 for control group 1, and $12.0 \pm 0.8\%$ at d 8 for control group 2, respectively. There was little difference between the peak parasitemias of the two control groups. Therefore, the non-specific immunity caused by the adjuvanticity of *L. lactis* was little. It should be noted that the appearance of parasitemia in the test group was one-day delayed compared with the control groups. None of the mice in any of the three groups died during the experiment. Overall, the BALB/c mice in all the three groups had the ability to scavenge *P. yoelii* parasites from their bodies by themselves.

For C57BL/6 mice, one control group and two test groups were designed. Mice were administered with 5×10^9 plasmid-harbored *L. lactis* cells per dose in test group 1, and 1×10^8 cells per dose in test group 2. Vaccination was performed by the same protocol for BALB/c mice, and the parasitemias were measured from the next day of parasite challenging. As shown in Figure 4A, the average peak parasitemias for the three groups were $60.8 \pm 9.6\%$ for the control group, $41.6 \pm 8.8\%$ for test group 1, and $20.8 \pm 26.5\%$ for test group 2, respectively. Surprisingly, test group 2 administered with 1×10^8 bacterial cells per dose gained significantly stronger immune protection than test group 1, which was administered with 15 times more bacterial cells per dose. However, the standard deviation

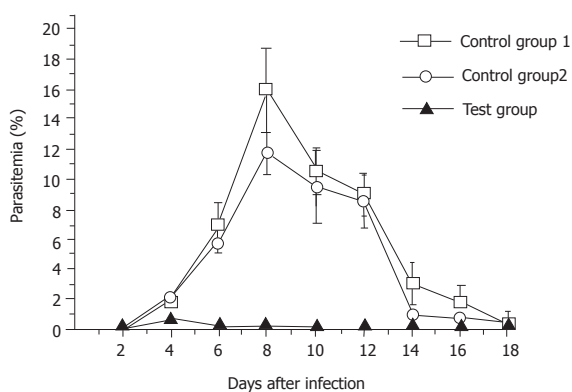


Figure 3 Blood-stage parasitemia of immunized BALB/c mice challenged with *P. yoelii* 265BY parasites. Mice were orally administered with PBS in the control group 1 (-□-), with 5×10^9 per dose of free *L. lactis* cells in the control group 2 (-○-), and with 5×10^9 per dose of *L. lactis* cells carrying pL2-PSGT construct in the test group (-▲-), respectively. Immunization procedure is described in Materials and Methods, and each mouse was challenged with 1×10^5 asexual blood-stage *P. yoelii* parasites.

of test group 2 was remarkably bigger than the other two groups. This was largely due to the difference between individuals. Another important fact that should be noted was that all the mice in the control group died within 23 d after parasite infection, whereas all the vaccinated mice survived despite the high parasitemias (Figure 4B). After 30 d counting from the day of parasite challenging, malarial parasites were no more detectable in both of the two test groups (data not shown), implicating the complete elimination of the *P. yoelii* parasites.

We also checked the duration of recombinant bacteria in mice gut by investigation of the titers of recombinant bacterial cells in mice feces. After feeding of a single dose of 5×10^9 recombinant cells, the recombinant *L. lactis* reached a peak at 6 h with a density of 1×10^7 cells/g of feces, and then gradually decreased with time. The density of recombinant cells decreased to 1×10^4 /g at 48 h, and 1×10^3 /g at 72 h. Therefore, the interaction between the host and recombinant bacterial cell could be as long as 3 d per dose.

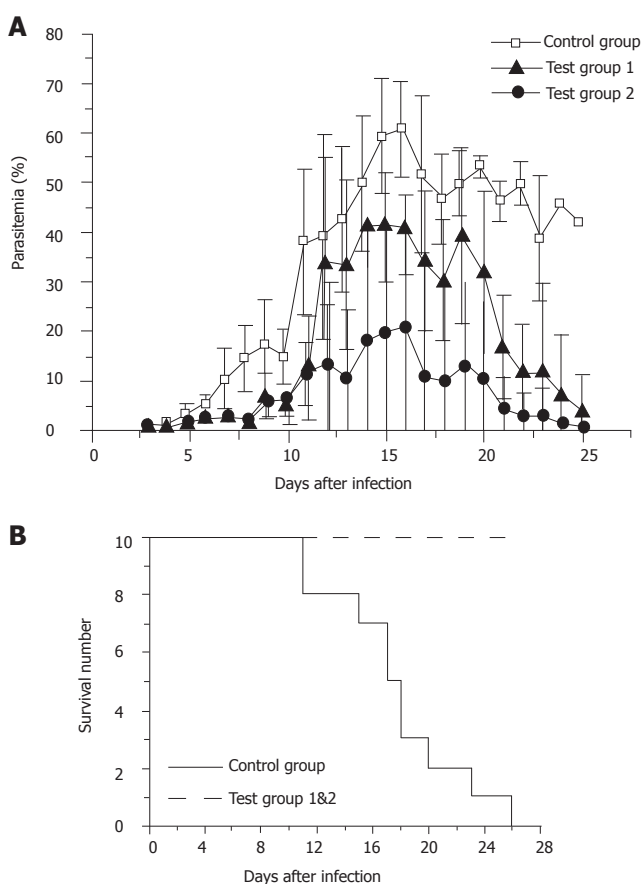


Figure 4 (A) Blood-stage parasitemia of immunized C57BL/6 mice challenged with *P. yoelii* 265BY parasites. Mice were orally administered with 5×10^9 per dose of free *L. lactis* cells in the control group (-□-), with 5×10^9 pL2-PSGT plasmid-harbored *L. lactis* cells per dose in the test group 1 (-▲-), and with 1×10^8 cells per dose of pL2-PSGT plasmid-harbored *L. lactis* cells in the test group 2 (-●-). Immunization procedure is described in Materials and Methods, and each mouse was challenged with 1×10^5 asexual blood-stage *P. yoelii* parasites. (B) Time course of survivals of the mice in the three groups is indicated in A.

DISCUSSION

It is known that the immunogenicity of soluble protein is low when administered orally but when expressed by genetically engineered bacteria and can be considerably enhanced. To achieve this goal, promoters that can drive the expression of a gene constitutively are essential. S-layer protein is a protein that forms regular crystalline arrays on prokaryotic cell surface. The *slpA* promoter can express the β -lactamase constitutively at a high level in *L. lactis*^[27]. However, it failed to express MSP-1₁₉ with high efficiency in this study. The expression of MSP-1₁₉ in *L. lactis* could be detected only by immunoblotting; therefore, it was estimated at the level of several nanograms per 10⁷ bacterial cells.

Unexpectedly, the low-level expression of MSP-1₁₉ in *L. lactis* was still able to elicit strong protection against *P. yoelii* infection on both BALB/c and C57BL/6 mice by oral administration. Although BALB/c mice seemed to be able to scavenge the malarial parasites by themselves, the parasitemia was reduced more than 10-folds (less than 1%) in the test group compared with the control groups. In the case of C57BL/6 strain, all the members in the control group died within 26 d after the infection, whereas all the members in the two test groups survived, and the parasites disappeared from both groups one month after the parasite challenging.

The different immune responses of the two C57BL/6 test groups also support the point of view that the expression level of MSP-1₁₉ is not critical for the elicitation of immune response, i.e., the group administered with a low dose of the recombinant bacterium gained stronger protection than the group administered with a high dose. The reason for the difference between the two groups is not clear at present; however, this might be partially due to immune tolerance caused by overdose. In the study by Robinson *et al.*^[21], expression of tetanus toxin fragment C of *C. tetanus* in the intracellular accumulation of *L. lactis* was up to 3% of soluble cellular protein, and 5×10⁹ cells were orally administered per dose to gain complete protection of mice from tetanus. Therefore, the optimal dose and the time schedule for oral administration should be carefully determined for each antigen.

It is striking to find the difference in immune response between the two strains of mice, BALB/c and C57BL/6. Our results partially support the report by Tian *et al.*^[28] that C57BL/6 mice are most sensitive to *P. yoelii* infection. In most cases, BALB/c and C3H/He mice are used for protection test of a vaccine. Our results suggest that at least for protection experiments, C57BL/6 mice are better to be used in parallel.

In vaccination experiments with recombinant MPS-1₁₉ of *P. yoelii*, the protection effect has been found to be mediated by humoral immune response^[15-17]. Robinson *et al.*^[21] also reported high titers of IgG against tetanus toxin. We also tried immunoblotting and ELISA test with sera from the survivals of C57BL/6 mice in the test groups (data not shown). Nearly all the mice generated IgG against MSP-1₁₉, and there were no significant differences between

the two test groups at the titers of IgG. In general, the titers were between 5×10² and 3×10³, lower than the titers reported by Robinson *et al.* On the other hand, Lee *et al.*^[23] measured the antigen-specific IgG titers in monkeys immunized with recombinant *L. lactis* bacterium expressing *H. pylori UreB* gene, and titers as high as 1×10⁵ were detected. However, despite the presence of high-titer antigen-specific IgG, all monkeys were infected after *H. pylori* challenge, and there were no differences in the density of colonization. Taking our results obtained from low-dose test group of C57BL/6, we suggest that high-titer antigen-specific IgG should not be considered as the major indicator of the protective immune response. The role of cell-mediated immunity played in live bacteria vaccine should be focused.

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