Immunogenicity of Oral Vaccine Candidate in Recombinant *Lactococcus lactis* Expressing HBcAg and IFN α -2b for Hepatitis B Prevention

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Abstract

Background: Hepatitis B is a liver inflammation caused by virus infection leading to acute and chronic conditions. Antigenic compound of HBcAg could induce specific T and B cell that generating high immunity response rather than HBsAg. Therapeutic agent for hepatitis and cancer treatment which has been approved by USFDA is Interferon α -2b (IFN α -2b). This type of class I cytokine plays a role in inhibiting viral replication and modulating adaptive immune system.

Objectives: In this study, we analyzed immunogenicity of recombinant HBcAg and IFN α -2b expressed in *L. lactis* NZ3900.

Methods: In vivo test was carried out using female Balb/c mice. Antibody responses for oral immunization were quantified as total IgG using ELISA on days 21, 35 and 51. Safety compound of this oral vaccine candidate was also described by liver and spleen condition after immunization. The differential leukocyte counting was performed to confirm the inflammatory process. **Results:** Post immunization with *L. lactis* recombinant strains could induce optimum total humoral immune responses on day 35, with IgG concentration at 4.96 ± 1.03 mg/mL. Single treatment with HBcAg was more potent in inducing immune response (IgG) rather than HBcAg-IFN α -2b combination. Immunization for 51 days could not alleviate animal body weight in each group. The maintaining IgG production until 51 days was just because lymphocytes activities persisted above 70%. The lymphocytes number which achieved 76.3% (P2) and 78.3% (P3) compared to Control group with 62%.

Conclusion: Single treatment with recombinant HBcAg expressed in *L. lactis* NZ3900 was better inducing IgG production and maintaining for 51 days. This result suggested, *L. lactis* recombinant strain can be as potential vaccine candidate to induce immune response protecting from hepatitis B virus. Moreover, no organ damage was found in liver and spleen of Balb/c mice.

Background

Hepatitis B Virus (HBV) is a major cause of hepatocellular carcinoma containing two main structural proteins, Hepatitis B Surface Antigen (HBsAg) and Hepatitis Core Antigen (HBcAg). HBV infection could lead into chronic condition which is a potential cause of cirrhosis and liver failure [1]. Indonesia was the second largest of hepatitis B endemicity in South East ASEAN Region (SEAR) subsequent to Myanmar, with the percentage of 2.5-10% [2,3]. Several hepatitis B treatments have been developed either for prevention or therapeutic effects. HBsAg was the first yeast-derived recombinant vaccine developed since 1982 and it had been decreased the cases of infection significantly. Furthermore, antiviral drugs such as lamivudine, adefovir, entecavir, tenofovir could suppress viral replication, but they could not eradicate the persistence of HBV in cccDNA form, and generate viral resistance for long-term treatment [4], [5]. This data indicated HBV infection should be handled through development of new vaccines that provide better antibody responses.

HBcAg has been reported as antigenic compound with high immunity which could induce specific T and B cell. The first antibody response (IgM) was specific to HBcAg, whereas antibodies specific to HBsAg and HBeAg were detected afterwards, and those imply a better prognosis with HBcAg [4]. Yet, *in vivo* tests of HBV vaccine from combination of HBcAg, HBsAg, and adjuvant (such as saponin-based ISCOMATRIX™, cytosine-guanine dinucleotide (CpG)) in transgenic mice, generated humoral and cellular immune responses, HBV specific T and B cell without any liver damage [6,7]. Considering all aspects, strong innate immune response is the crucial factor to evoke better effect in eradicating HBV. Type I interferons (IFN α and IFN β) are main effector of innate immunity, therefore United States Food and Drug Administration (USFDA) approved Interferon (IFN) α -2b as therapeutic agent and 86 countries have been used it for hepatitis and cancer treatments [8]. Currently, vaccine administration was through intranasal or intravena, which was high cost limit and complicate challenges in developing country with dense populations, such as Indonesia. Heretofore, developing an inexpensive and easily administered vaccine is important for HBV treatment.

Lactococcus lactis is gram-positive bacteria and had been used for antigen delivery system through mucosal routes. Recombinant *L. lactis* used to express protein or antigen has several advantages, such as no-endotoxins produced in *L. lactis*, non-pathogenic and non-colonizing characteristic in mucosal tract [9]. Various recombinant antigen expressed in *L. lactis* had been reported, e.g. nucleocapsid protein of SARS-coronavirus, HIV enveloped-protein, E-domain III of Dengue virus, and they have been tested for intra-vena or oral administration [10]. Moreover, recombinant HBsAg protein has been reported in *L. lactis* NZ9000 and generated humoral (IgG) and mucosal (IgA) immune response in female Balb/c mice [11]. In Indonesia, dominant detected HBV was sub-genotype B3 in Java Island [12,13]. Additionally, our previous work has been successfully cloned HBcAg of thus sub-genotype into food-grade *L. lactis* NZ3900 and expressed extracellularly with NICE expression system [14]. However, *in vivo* test of recombinant HBcAg in *L. lactis* is still limited. Heretofore, in this presence study we reported the immunogenicity of recombinant HBcAg and combined with recombinant IFN α -2b expressed in *L. lactis*, which is expected to induce high immune response in female Balb/c mice.

Material And Methods

Bacterial and Animal Strains

L. lactis NZ3900 was a host strain used in this study for control treatment. Recombinant HBcAg and IFN α -2b that was successfully cloned into *L. lactis* using plasmid pNZ8148 [14,15] was obtained from Biotechnology Research Center, LIPI, Cibinong. Specific Pathogen-Free female mice BALB/c 18-25 g (7-8 weeks old) were conducted from Bogor Life Science and Technology (BLST)-IPB University. Procedures related to animal raising and specimen sampling had been received ethic approval from the Animal Ethic Commission decree, number 114-2018 IPB. The guideline refers to PKBPOM No. 17 year 2015.

Inocula preparation for immunization

To confirm recombinant strain used for immunization, *L. lactis* pNZ8148-HBcAg and IFN α -2b was analyzed by PCR colony [16] using PnisA (promotor) and TpNZ8148 (terminator) primer. PCR condition for 35 cycles was carried out as follows: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 30 s, followed by a final elongation at 72°C for 6 min. PCR product was confirmed in 1% agarose with ethidium bromide staining.

Host and recombinant *L. lactis* were cultured on M17 enriched with 0.5% glucose and cultivated on 30°C without shaking. Antibiotics and inducers nisin were added to recombinant strains as follows: chloramphenicol (10 mg/ml) and nisin (10 ng/ml). Nisin were added when strains growth until OD₆₀₀ ~0.5. After induction, strains were incubated for 45 minutes until OD₆₀₀ ~0.6. Pellet cells were harvested by centrifugation 3000 x g at 4°C for 10 minutes and washed three times with sterile PBS 1x [17]. Pellets were suspended in PBS 1x to a final concentration of 10¹⁴ CFU. Total plate counts were implemented to all inoculum to confirm CFU administered for immunization.

Mice immunization and sample collection

Mice were housed with controlled room temperature (27-28°C) and sterile water *ad libidum* in the Educational Animal Hospital, Veterinary Faculty of IPB University. Three groups of mice (5 mice each group) immunized with 10¹⁴ *L. lactis* NZ3900 (NC), 10¹⁴ induced recombinant *L. lactis* HBcAg (P1), and 10¹⁴ induced recombinant *L. lactis* IFN α -2b (P2). One group (P3) of other mice immunized with 10¹⁴ induced recombinant *L. lactis* HBcAg and IFN α -2b (1:1) suspended in 0.1 ml sterile PBS 1x and administered orally on day 0, 1, 2, 14-16 and 28-30 [11]. The treatment groups are presented in Table I. Animal body weights were measured before immunized and 7 days after immunization. Serum samples were collected on day 21, 35 and 51. Animal sacrifice was on day 51, and collection of liver and spleen for histopathology analysis.

ELISA

Quantification of total IgG in serum were analyzed using protocol of protein science, InnoBio. ELISA plates (Costar, Corning Incorporated, NY, USA) were coated with 1 μ g/mL IgG capture M8645 in 100 μ L 0.1 M sodium carbonate-bicarbonate (pH 9.6) per well overnight at 4°C. Blocking with PBS 1x containing 10% skim milk and 0.02% Tween 20 was carried out at 37°C for 90 min. Serum samples (100 μ L per well) were added as primary antibodies in triplicate at 1:16000 dilution with PBS 1x. IgG standard was added for serial concentration at 500 ng/ml to 3.9 ng/mL. The plates were then incubated at 37°C for 90 min following three times washes with 200 μ L aquabidest containing 0.09% NaCl and 0.05% Tween 20. Bound antibodies were detected using HRP-conjugated IgG γ -chain (1:2500) and then incubated at 37°C for 90 min. After washing, ABTS substrates were added 100 μ L per well and measured at 405 nm. Standard absorbances were plotted to determine equation for IgG concentration.).

White Blood Cells differential count

The cells were counted using a 40x objective in a strip running the whole length of the film. The film was inspected from the head to the tail and if fewer than 100 cells are encountered in a single narrow strip, one or more additional strips were examined until at least 100 cells have been counted. Each longitudinal strip represents the blood drawn out from a small part of the original drop of blood when it has spread out between the slide and spreader [18].

Liver and spleen histopathology

To determine safety of recombinant vaccine candidate, we analyzed liver and spleen histopathology in the Primate Animal Study Center, IPB University. Liver and spleen tissue specimen were fixed in Buffer Neutral Formalin (BNF) and embedded in paraffin. 10 µm sections of tissue were stained with haematoxylin and eosin (HE) and analyzed descriptively by pathologist. The analysis was emphasized to the presence of mononuclear infiltration cell in the tissue.

Statistical analysis

Animal body weight changes for 51 days treatment was analyzed using two-way Anova. The antibody responses (IgG) of immunized mice were measured as absorbance which converted into concentration (ng/ml). The statistical significances were analyzed using One-Way Anova and Tukey post-hoc analysis in SPSS 16.0 program.

Results And Discussion

One of media for HBV spreading out was from body fluids, e.g. saliva, menstrual, vaginal, and seminal [1]. Most pathogens initiate infection through mucosal site, so early immunization from mucosal tract can increase immunity against infectious agents [19]. In this study, we had been tested immunogenicity of recombinant strain of *L. lactis* containing HBcAg and IFNα-2b in female Balb/c mice. The confirmation of recombinant strain used for immunization through colony PCR, generating confirmed positive band of three colony picked from each strain in Fig. 1. Plasmid without insert gene was confirmed at 440 bp in length (Fig. 1A). The recombinant HBcAg was amplified in ~1080 bp, and IFNα-2b with ~1033 bp. The native HBcAg gene construction in pNZ8148 was 630 bp length, and codon optimized of IFNα-2b was 579 bp in length [14,15]. The insert genes were persisted in the Multi Cloning Site (MCS) of pNZ8148 (Fig. 1B).

Four groups were immunized orally with control strain *L. lactis* NZ3900 and recombinant strains for three times booster in sequence. In day 21, no significant differences of total IgG in each group, and significantly elevated IgG in day 35 for all groups (Table II). In day 51, IgG change trends with significant difference was pointed out by Control ($P=0.036$) and P1 ($P=0.044$) groups. This statistical analysis result was assumed that Control and P1 groups may be lack to maintain the optimum IgG production up to 51 days, compare to groups which treated with IFNα-2b. Moreover, P3 generated lower IgG concentration compared to HBcAg (P1) or single IFNα-2b (P2) on day 21-51. This data indicated that single treatment with recombinant HBcAg was better to induce high IgG production. To this result, the study reports of

exogenous HBcAg was more efficiently presented by B cell rather than other primary antigen presenting cells (APC), e.g. dendritic cells (DCs) [20,21]. The stimulated and activated HBcAg specific B cells could directly differentiate to be mature plasma cell secreting antibodies. Immunogenicity of HBcAg in this study represented as a strong immunogen even without adjuvant. Three dimensions structure of HBcAg was unique with regular spacing between repetitive spikes on its surface. To those unique features, HBcAg could induce high number of naïve B cells. The HBcAg-membrane receptor binding could generate intracellular signaling, secretion of IgM and IgG. Furthermore, co-administration of HBcAg and HBsAg has unique effect, while HBcAg could act a potent Th1 adjuvant to HBsAg, besides as immunogenic target [22].

To elicit immune response, several strategies had been developed, such as using of cytokine as adjuvant, e.g. interferon and interleukin [23]. Immunomodulatory effect of IFN α -2b was through activating JAK-STAT pathway and initiated the expression of ISRE regulator gene related to immune regulator protein, e.g. MHC-I [24]. The spontaneous immune responses have been shown depend on the activation of DCs by type I IFNs. Since DCs was reported the most professional APCs, so using proper dose of IFN α -2b for some immunotherapies in DCs activation was so crucial. DCs will act to capture antigen and priming to T cells and activated the naïve B cell to produce antibodies. Regarding to its ability, the dose of recombinant IFN α -2b used in this study may not enough to activate more the immunomodulatory pathway and generate higher IgG production, while combining with efficient immunogen, HBcAg. Moreover, this study also in line to the previous report that explained the innate immune system which may act minor rule compare to immunogenic antigen like HBcAg [20]. Another report showed the co-administration of recombinant HBsAg strain and IFN α -2b enhanced HBsAg specific antibody ~2 fold higher than single HBsAg [11]. To this data, treated group with recombinant strain may still need more additional dose in enhancing IgG production for 51 days treatment. Furthermore, as a control strain, *L. lactis* NZ3900 also induced better total IgG production compared to the other groups. Lactic Acid Bacteria (LAB) had been proven as immunomodulator based on dose and period of administration [25]. In this study, 10^{14} CFU *L. lactis* NZ3900 could induce total IgG production in serum.

To address the immunity process, we analyzed the differential counts of White blood cells (WBCs). They are heterogenous nucleated cells found in circulation, play a main role of immunity and phagocytosis. WBCs are classified into lymphocytes, monocytes, and granulocytes (eosinophils, basophils, neutrophils) [26]. To determine the number of each WBC on day 51, as the final treatment of immunization, we analyzed differential of leukocyte as the percentage of each type of cell. This analysis related to the total leukocyte count and pointed to analyze inflammatory process. Referring to IgG concentration, P2 and P3 group did not significantly decrease in day 51. This data could be related to lymphocytes number which achieved 76.3% (P2) and 78.3% (P3) compared to Control group with 62% (Table III). Maintaining IgG production until 51 days may be caused by lymphocytes activities persisted above 70%, which were analyzed on the last treatment day. Eosinophils and basophils are not circulating, but they act for limit inflammation in the tissue, so there was no detected this kind of leukocytes (Table III). Lymphocytes are known as fundamental cell in cellular and humoral immunity represented a normal percentage at 20-36%

in the blood [18]. From this data, co-administration with IFN α -2b could induce more proliferation of lymphocytes B which is responsible to synthesis antibody (IgG) and maintaining this condition for 51 days treatment. Single treatment with 10¹⁴ recombinant HBcAg persisted the IgG production optimum for 35 days and alleviated the IgG concentration on day 51.

The safety compound of oral vaccine candidate used in this study was analyzed through animal body weights at day 0 and 7 days after immunization (Fig. 2). Statistical significance confirmed weight gain 7 days post-immunization than initial body weight. Oral immunization with recombinant *L. lactis* do not affected the animal appetite, so there are no decreasing body weights after immunization. Additionally, there is no sign of ill-health within the period. Vaccine candidate has non-toxic effect as follows criteria : a) the animal body weight in 7 days after immunization are not less than initial weight, b) animal death during the testing period is not more than 5% and does not cause pain after vaccination [19,20]. Safety compound of vaccine candidate was also described by tissue and organ condition after immunization. As induction of liver inflammation was the major concern of HBV therapeutic vaccine, so here we reported analysis of adverse effect on liver tissue by descriptive histopathology analysis based on the presence of inflammatory cell. In the Fig. 3, there were pointed number of mononuclear cells at mild to moderate level. This infiltration was related to lymphocytes aggregates, plasma cell and macrophage in the tissue [29]. The infiltration in all groups was compared to naïve liver tissue of Balb/c mice which generated slight difference. Immunization with *L. lactis* up 51 days did not generate liver damage to necrosis stage, whereas the period of immunization was classified to chronic immunization up to 90 days treatment [30]. The result indicated that oral vaccine candidate used in this study was not cause liver damage and no adverse effect to liver tissue during treatment. In addition to determine activities of lymphoid system responded to oral vaccine candidate used in this study, we reported spleen histopathology as well. Spleen was the second lymphoid organ and host of all mononuclear phagocyte types i.e. macrophage, dendritic cells and monocytes. As a hosting organ of circulated T and B cell for antigen presentation, spleen played a vital role reacting of any antigen exposure [25]. Spleen histopathology in the Fig. 4 described no abnormalities detected of all groups compared to naïve spleen tissue. The result of histopathological description was presented on Supplementary Data 1.

Conclusion

Recombinant *L. lactis* containing HBcAg has better immunogenicity rather than combined with IFN α -2b as adjuvant. Treatment group with IFN α -2b generated prolonged lymphocytes proliferation up to 51 days. The expressed HBcAg in *L. lactis* could induce optimal humoral immune response in day 35 without organ damage. Additionally, oral vaccine candidate used in this study was safe, by no lowering body weight for 51 days treatment, implying that recombinant *L. lactis* HBcAg could be alternative vaccine candidate with strong immunogenicity and easily administered for HBV prevention.

Declarations

Acknowledgement

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Contributions

AZM and LM planned, designed the research, wrote the manuscript and performed experiments. SB, HSD, APM planned and designed the research. LT, AK, LS and DU performed experiments and analyzed data. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

This article had been received ethic approval from the Animal Ethic Commission decree, number 114-2018 IPB.

Informed consent

All authors gave their consent for the publishing of the manuscript.

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Tables

Table 1. Treatment Groups

Group	Treatment
Control	<i>L. lactis</i> NZ3900
P1	Recombinant <i>L. lactis</i> pNZ8148-HBcAg
P2	Recombinant <i>L. lactis</i> pNZ8148-IFN α -2b
P3	Recombinant <i>L. lactis</i> pNZ8148-IFN α -2b+HBcAg

Table 2. Total IgG in serum of Balb/c mice

Group	IgG Concentration (mg/mL)		
	Day 21	Day 35	Day 51
Control	1.84 $\pm$ 0.332 ^a	6.23 $\pm$ 0.399 ^b	5.48 $\pm$ 0.124 ^a
P1	1.78 $\pm$ 0.112 ^a	4.86 $\pm$ 0.105 ^{ab}	4.18 $\pm$ 0.393 ^{ab}
P2	1.64 $\pm$ 0.087 ^a	5.05 $\pm$ 0.624 ^{ab}	4.86 $\pm$ 1.111 ^{ab}
P3	1.39 $\pm$ 0.227 ^a	3.71 $\pm$ 0.879 ^a	3.56 $\pm$ 0.789 ^a

^aThe numbers in the same column followed by different subset letters are significantly different at a test level of 5% (Tukey interval test).

Table 3. Differential counts of WBCs in serum

Leukocyte types	Groups		
	<i>Control</i>	<i>P2</i>	<i>P3</i>
Lymphocytes	62.00±6.57 ^a	76.33±1.53 ^a	78.33±1.15 ^b
Monocyte	3.33±2.31 ^a	1.67±0.58 ^a	0.33±0.58 ^a
Eosinophils	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
Basophils	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
Neutrophils	34.67±4.51 ^a	22.00±1.73 ^b	21.33±0.58 ^b

Figures

Figure 1

Confirmation of recombinant *L. lactis* strain of HBcAg and IFNα-2b. (Left) Electrophoregram of PCR product, 100 bp DNA Marker, M (Vivantis). (Right) Plasmid map of pNZ8148 (Mobitec).

Figure 2

Balb/c mice body weights for 51 days treatment. Different subset letter showed significance at 5% level (Tukey interval test).

Figure 3

Liver histopathology of Balb/c mice after immunization with *L. lactis* in magnitude (20x). (a) Naïve liver; (b) Control; (c) P1; (d) P2; (e) P3. Broken line was represented infiltration area in the tissue.

Figure 4

Spleen histopathology of Balb/c mice after immunization with *L. lactis* in magnitude (20x). (a) Naïve spleen; (b) Control; (c) P1; (d) P2; (e) P3.

Supplementary Files

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