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ORIGINAL ARTICLE

Immunogenicity assessment of Hepatitis A-VP1 and Hepatitis B surface antigen (HBsAg) fusion protein: a novel bivalent vaccine candidate

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ABSTRACT

Background and Objectives: Subunit vaccines have the privilege of utilizing immunogenic parts of the variable viruses. The current preventive vaccines against Hepatitis A are based on live-attenuated virus or wild-type growth in cell culture, which is a time-consuming and costly procedure. Thus, the investigation of immunogenic Hepatitis A Virus (HAV) regions seems to be a rational priority. We aimed to evaluate a novel chimeric protein composed of truncated HAV-VP1 and Hepatitis B surface antigen (HBsAg) as a bivalent vaccine candidate in BALB/c mice.

Materials and Methods: The HAV-VP1 (amino acids 99 to 259) and HBsAg fusion protein were applied as a bivalent vaccine in combination with adjuvants. The purified protein was administered through different regimens via subcutaneous injection. Two weeks following the final immunization, serum samples were gathered to assess the humoral responses. Moreover, splenocytes were investigated and assessed for IL-5 and IFN-γ secretion.

Results: The immunized mice with recombinant truncated HAV-VP1-AAY-HBsAg showed a significant immune response, especially in combination with the M720 adjuvant. Humoral immune response results indicated Th1 switching by IgG2a and IgG2b dominancy. Moreover, IFN-γ secretion reached the highest rate in the truncated HAV-VP1-AAY-HBsAg+M720 recipients ($p<0.0001$).

Conclusion: The HAV-VP1-AAY-HBsAg protein subunit vaccine could help the immune system fight HAV and HBV by stimulating both the humoral and cellular immune systems. The formula proposed in this study has the potential to produce an endemic vaccine based on the circulating HAV viruses in Iran.

Keywords: Subunit vaccine; Hepatitis A; Hepatitis B; Bivalent vaccine; Hepatitis A virus VP1 protein; Adjuvant

INTRODUCTION

Hepatitis A virus (HAV) is one of the five viruses identified as causing viral hepatitis and is the leading reason for acute viral hepatitis cases (1-3). As a single-stranded RNA virus from the *Picornaviridae* family, HAV spreads largely through the fecal-oral route when in contact with those who are infected, resulting in liver inflammation and harm to liver cells (4, 5). Nearly 200 million people are affected by this infection each year, and it results in approximately 30,000 deaths (6-8).

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In a massive study conducted in Mexico from 2000 to 2019, an average 14.7 HAV cases per 100,000 inhabitants were reported annually. Although the highest incidence rate belonged to children <9 years, the hospitalization rate associated with HAV infection showed an increase among older groups (9). Therefore, this may consequently increase the risk of complications and severity in the older population, which demands healthcare resources. There are no approved therapeutic agents for hepatitis A except supportive care. Thus, vaccination can bring an enormous contribution to preventing hepatitis A, as it is always recommended as the most practical method against infectious diseases (10-12). For many years, vaccines that ensure both safety and effectiveness have been created and used. Consequently, numerous countries have implemented widespread vaccination for children, yet this practice is not as common among high-risk adults (13, 14). Hepatitis B virus (HBV) is another significant cause of liver inflammation besides hepatitis A and often leads to long-lasting infections. In areas where the virus is common, HBV spreads mainly through contact with infected bodily fluids, particularly blood (15, 16). According to WHO estimates, 296 million individuals globally are affected by HBV. Hopefully, the rate of chronic infection with HBV in children declined to <1% in 2019 in the pre-vaccine era (17-19). Recombinant vaccine platforms have been successfully established against HBV, which mainly stem from viral surface antigen (HBsAg) (20, 21).

Contrary to the Hepatitis B vaccine platform, the vaccines against HAV include live attenuated and inactivated platforms with a successful impact on vaccine coverage. HAV presents lower rates of the first and second doses (87% and 57%) in comparison with other childhood vaccinations (22). Recombinant protein vaccines have been shown to be immunogenic and safe with the potency of immune response induction against the conserved epitopes of mutable viruses (23, 24). Prevention from HAV and HBV infections in a single combined platform through a single injection has been taken to attention. This development has not only led to widespread public satisfaction but has also been well-received in terms of its immunogenicity profile in both the USA and Europe (25). HAV capsid protein is composed of 60 VP1-VP4 copies, which is the main target in vaccine design (26). The VP1 plays an immunodominant role in neutralizing antibody generation, and so it could be investigated in prophylactic vaccines. It has been shown that VP1 consists of interacting sites with neutralizing antibodies (27, 28). The most recent HAV and HBV bivalent vaccines are based on live attenuated HAV and recombinant HBV combinations. Hence, a single vaccination could bring the hope of hepatitis A and B elimination if considered globally. In other words, a bivalent recombinant protein-based vaccine against HAV and HBV seems an ideal platform to elicit the immune system against both pathogens. For instance, Ahmadi et al. designed a bivalent vaccine containing epitopes of HAV-VP1 capsid protein and HBsAg using an immunoinformatic approach and reverse vaccinology in which proteins were fused with an AAY (Ala-Ala-Tyr) linker. Their predictions revealed that the VP1-AAY-HBsAg fusion protein could be a promising candidate vaccine against infection with HAV and HBV (29). The AAY linker in multi-epitope vaccines not only acts as the cleavage site for proteasomes, but also improves the stability and immunogenicity of vaccines (30, 31). In our previous study, we successfully expressed a newly developed recombinant protein incorporating fused domains comprising a highly conserved region within the HAV-VP1 protein encompassing amino acids 99 to 259 and entire of HBsAg designed as a bivalent vaccine candidate targeting both infections (8). Herein, we hypothesized that the previously designed and constructed bivalent HAV-VP1-AAY-HBsAg vaccine could induce humoral immune responses against HAV and HBV; therefore, we assessed the immune response induced by the bivalent vaccine in combination with different adjuvants against HAV and HBV in a mice model.

MATERIALS AND METHODS

Ethic statement. Ethical approval for the research was granted by the Ethics Committee of the Pasteur Institute of Iran (No#IR.PII.REC.1398.053).

Design and expression of HAV-VP1-AAY-HBsAg construct. The bivalent HAV-VP1-AAY-HBsAg vaccine was designed and evaluated according to bioinformatics tools for their antigenicity, allergenicity, toxicity, and ability to bind to B lymphocytes and T lymphocytes. Moreover, the primary, secondary, and tertiary structure of the vaccine were assessed and validated (8). The constructed bivalent HAV-VP1-

AAY-HBsAg vaccine was cloned and expressed as described previously. Briefly, pET-24a containing HAV-VP1-AAY-HBsAg was cloned into *Escherichia coli* DH5α strain and then expressed in *E. coli* BL21(DE3). The truncated VP1-AAY-HBsAg as a ~ 46 kDa protein was finally expressed, purified, and prepared for injection (8).

Mice immunization. Female BALB/c mice, between six and eight weeks of age, were acquired from the Pasteur Institute of Iran. Forty mice were assigned using randomization method into eight categories for experimental analysis (n = 5 per group), presented in Table 1. The injection was done via a subcutaneous route into the dermal looseness neck on three occasions, spaced two-week apart (0, 3, and 6). Two primary control groups were established: HBsAg and VAQTA (the inactivated Hepatitis A vaccine). In the formulations with alum-adjuvants, alum hydroxide was combined with the antigen in a 1:1 volume ratio, while the Montanide-adjuvanted vaccines were created using a 30:70 volume ratio of aqueous to oil.

Antibody response assessment with enzyme immunoassay (ELISA). Blood was drawn via the retro-orbitals plexus two weeks following the final injection. Then, serum samples were isolated to store in -80°C after thermal-inactivation. Antibody responses post-vaccination were assessed using an indirect ELISA. Briefly, 100 µl (3 µg/ml concentration) of truncated VP1+HBsAg protein was immobilized onto a pair of 96-well microplates (Nunc, Denmark) and incubation under refrigeration (4°C) was conducted for 16 hours. In the next step, a volume of 100 µl of each serum sample, diluted at 1:10000 was introduced to the designated well. After incubating at 37°C for 60 minutes, the wells underwent wash and block steps before adding 100 µl of Goat anti-mouse total IgG-HRP antibodies at a dilution of 1:10000 (Sigma-Aldrich, USA) for another hour of incubation. In the end, 100 µl of TMB substrate (3,3′,5,5′-Tetramethylbenzidine; Sigma-Aldrich, USA) was introduced and incubated at ambient temperature. Optical density at 450 nm was measured. Additionally, the subclasses of IgG antibodies, namely IgG1, IgG2a, and IgG2b were monitored using 1:2000 concentration of antibodies (Sigma-Aldrich, USA) following the same porotocol.

Cytokine release assessment with ELISA. Murine ELISA kits were utilized to measure IFN-γ and IL-5, following the guidelines provided by the manufacturer (U-CyTech Biosciences, Netherlands). To collect spleens, both the vaccinated and control mice were euthanized. The spleens were then processed using a cell homogenizer to create a suspension of splenocytes. Afterward, RBC lysis buffer was applied to these cells, which were subsequently washed using phosphate-buffered saline (PBS) for 10 minutes at 4°C. Ultimately, a concentration of 2 × 10^6 cells/ml was seeded into 24-well plates (Nunc, Denmark) that contained Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Germany) enriched with 10% fetal bovine serum supplement (FBS; Gibco, Germany), 100 mg/ml streptomycin (Biosera, USA), 100 U/ml penicillin, and 2 mM L-glutamine (Biosera, USA). This setup was maintained for a 72- hour period with 10 µg/ml of the truncated VP1 + HBsAg fusion protein. An ELISA reader (Bio-Tek, USA) was then used to measure the optical density of the wells at 450 nm.

Table 1. Immunogenicity of mice

Group No.	Agent	First Injection	Booster
1	PBS	PBS	PBS
2	Montanide	Montanide	Montanide
3	Alum	Alum	Alum
4	VAQTA*	VAQTA	VAQTA
5	HBsAg	HBsAg	HBsAg
6	Truncated** VP1+HBsAg	Truncated VP1+HBsAg	Truncated VP1+HBsAg
7	Truncated VP1+HBsAg +Alum	Truncated VP1+HBsAg +Alum	Truncated VP1+HBsAg +Alum
8	Truncated VP1+HBsAg +Montanide	Truncated VP1+HBsAg + Montanide	Truncated VP1+HBsAg + Montanide

* Hepatitis A Vaccine, Inactivated

**Truncated VPI contains amino acids from 99 to 259.

Data analysis. All data analyses were conducted with GraphPad Prism statistical software, utilizing One-way ANOVA and Mann–Whitney non-parametric tests. The results were shown as Mean ± SD. A p-value of > 0.05 was determined to be significant. Each experiment was performed in triplicate.

RESULTS

Humoral response assessment in vaccinated mice. Total IgG concentration and subclasses of IgG are presented in Figs. 1 and 2. All vaccinated mice with different regimens generated significant total IgG against truncated VP1+HBsAg in comparison with the controls ($p<0.0001$). According to Fig. 1, the highest level of IgG titer belonged to the truncated VP1+HBsAg + Montanide 720 group ($p<0.0001$). Moreover, regarding applied adjuvants, Montanide 720 led to a significant antibody induction ($p<0.0001$). Truncated VP1+HBsAg + Alum injection led to the highest amount of IgG1, followed by the truncated VP1+HBsAg + Montanide 720 group (Fig. 2). The IgG2a and IgG2b titers were higher in immunized mice with the truncated VP1+HBsAg + Montanide 720 group ($p<0.0001$), which was significantly comparable with other regimens (Figs. 2B and C).

In the evaluation of the humoral response, it was found that IgG2b was the most prevalent, suggesting a preference for Th1 switching. Furthermore, the truncated protein, in combination with Montanide 720, resulted in a remarkable immune response. According to the humoral immune assessment, the newly formulated vaccine candidate showed significantly remarkable antibody induction in comparison with VAQTA or HBsAg recipients ($p<0.0001$).

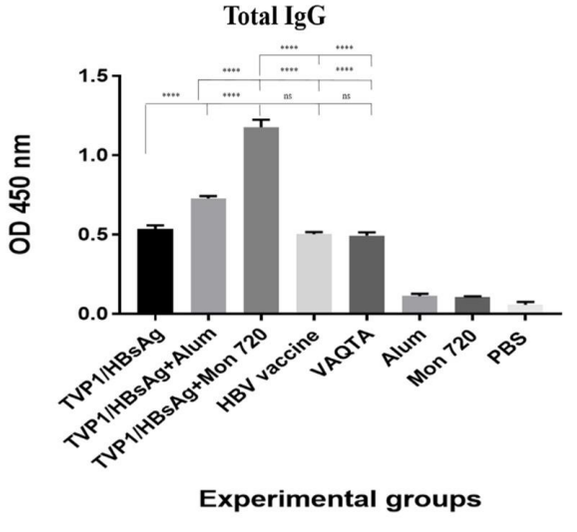

Fig. 1. Indirect ELISA of total IgG for analysis of humoral responses in sera of immunized mice. The data are presented as the mean ± standard deviation (SD) ($P<0.05$*, $p<0.0001$****).

Cytokine release assessment. The Th2 response was examined with a focus on IL-5, and its levels were compared across different groups. As shown in Fig. 3A, the use of the adjuvanted protein combined with Alum led to a notable increase in IL-5 levels, significantly higher than those from other formulations ($p<0.001$). Moreover, the cellular immunity response was evaluated by measuring the secretion of IFN-γ, as illustrated in Fig. 3B. The group that received truncated VP1+HBsAg + Montanide 720 demonstrated the highest secretion of IFN-γ ($p<0.0001$), which was also accompanied by the production of IgG2a and IgG2b, indicating a robust cell-mediated immune response. Additionally, Montanide M720 resulted in a greater induction of IFN-γ compared to the protein that was not adjuvanted ($p<0.0001$). Aligning with the findings on the humoral immune response, the truncated VP1+HBsAg induced a significantly stronger immune response when compared to the HBsAg or VQATA groups ($p<0.0001$).

DISCUSSION

Although strategies approaches have played a key role in the development of vaccines aimed at controlling viral hepatitis, for example inactivated vaccines against Hepatitis A, they are costly and time-consuming and this procedure requires virus generation and purification (32-34). Thus, subunit protein-based vaccines against hepatitis A seem to be rational approaches and also a priority against HAV infections (35, 36). Moreover, protein-based bivalent vaccines could provide protection against infections with both HAV and HBV in a single administration, reducing the cost and time of vaccine production (37). Bivalent vaccines also increase the satisfaction for the healthcare system by reducing injection numbers and medical visits, leading to an increase in adherence to vaccination schedules (38). In this research, we tailored and constructed a fu-

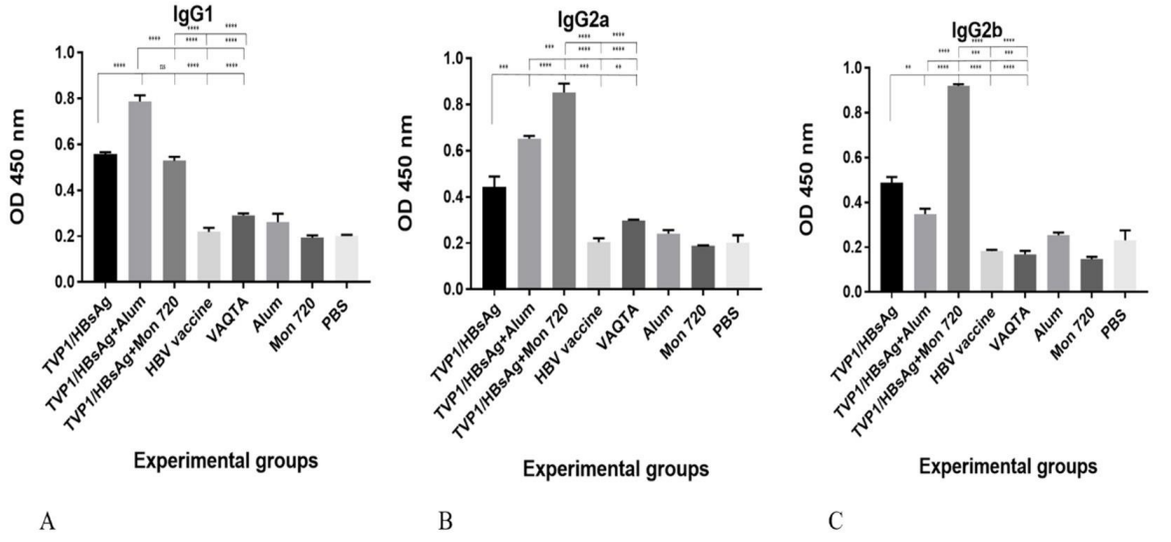

Fig. 2. ELISA of IgG1 (A), IgG2a (B), and IgG2b (C) isotypes in sera of immunized mice. The data are presented as the mean ± standard deviation (SD) (P<0.05*, p <0.005**, p <0.0005***, p <0.0001****).

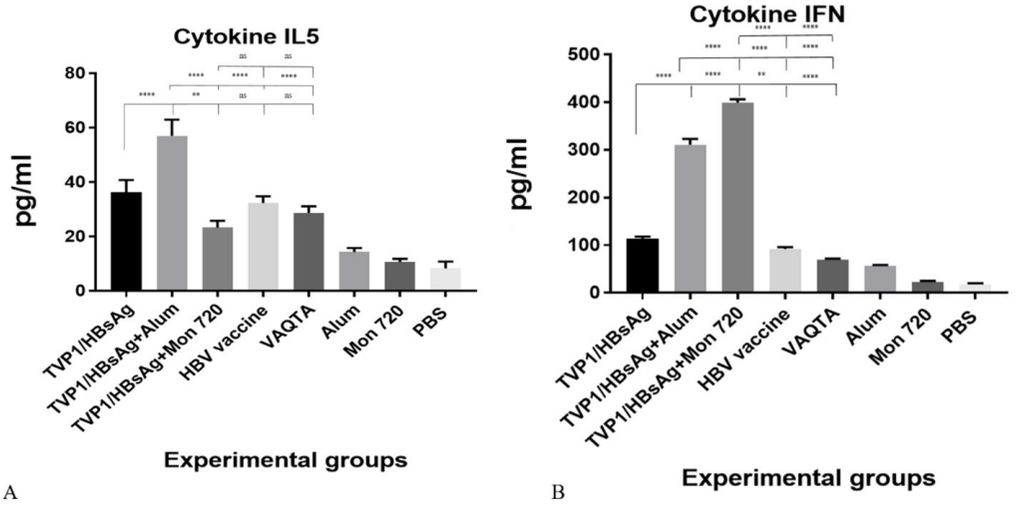

Fig. 3. ELISA analysis of IL-5 secretion (A) and IFN-γ secretion (B) on splenocytes of mice immunized by various formulations. The data are presented as the mean ± standard deviation (SD) (P < 0.05*, P < 0.005**, P < 0.0005*** and P < 0.0001****).

sion protein composed of an immunogenic region of HAV-VP1 protein (amino acids from 99 to 259) fused to the entire of HBsAg to evaluate its immunogenicity in combination with different adjuvants as a vaccine against hepatitis A and B in BALB/c mice. The findings revealed significant humoral and cellular induction for the novel truncated protein, especially in combination with the Montanide M20 adjuvant.

Despite the importance of Th1 responses (cell-mediated immunity) in controlling intracellular infections, such as infections with viral pathogens, Th2 responses (humoral immunity) are also responsible for controlling extracellular infections. Therefore, the induction of humoral responses, besides cell-meditated immunity, is vital in designing vaccines to control infection with hepatitis viruses (39). There are four distinct classes of IgG in mice, including IgG1, IgG2a, IgG2b, and IgG3, among which IgG1 contributes to the induction of Th2 response and the others are involved in Th1 profile (40). The dominan-

cy of IgG2a and IgG2b highlights the Th1 switching as well as IFN-γ secretion as a result of cellular immunity induction. Our finding demonstrated that the chimeric protein (truncated VP1+HBsAg) has potent immunogenic capabilities in BALB/c mice. This was supported by the production of specific IgG antibodies and the release of IFN-γ. Additionally, our data suggested that this chimeric protein's ability to trigger an immune response is better than that of the HBsAg vaccine or the inactivated HAV vaccine. The recombinant form of HAV-VP1 and its immunogenicity was studied by Lee et al., in which they showed that specific IgG in sera and IgA secretion in the small intestine were detected after intraperitoneal (i.p.) and oral administration, respectively. According to their reports, the secretory form of recombinant VP1 has the potency as an immunogenic agent to develop as a HAV vaccine (41). In a different research project, Jang et al. created and tested the effectiveness of recombinant VP1-His, VP1-3N-His, and 3D2-His vaccines in mice following an intraperitoneal injection. This vaccination led to the production of specific IgG antibodies targeting the proteins, and levels of IL-6 and IFN-γ in splenocytes were also monitored. The evaluation of IgG isotypes showed that IgG1 was the most common, with IgG2a and IgG2b coming next (39).

There is evidence that IFN-γ exerts its anti-viral functions through various mechanisms, including inhibition of virus entry, gene expression, virus replication, impairment of virus assembly and stability, and suppression of virus release and reactivation (42). In the case of viral hepatitis, certain immunosuppressive proteins, such as transforming interleukin-10 (IL-10) and growth factor-β (TGFβ) reduce the ability of natural killer (NK) cells to produce IFN-γ. This reduction allows hepatitis viruses to remain in the body (43). The rise in IFN-γ expression shows the possibility of a Th1 response, leading to a cytotoxic T-cell reaction and the clearing of the virus. IFN-γ is crucial for the T-cell response mediated by VP1, as an increase in IFN-γ levels is linked to Th1-type immunity. The VP1 protein of HAV as an immunogenic part of the virus has been investigated in some vaccine platforms. Chung et al. investigated the immunogenicity of HAV VP1-Fc as a recombinant protein. This protein consisted of HAV-VP1 and an Fc antibody fragment. The immunization of mice with HAV VP1-Fc elicited IL-4 and IFN-γ expression in splenocytes. Moreover, the HAV VP1-Fc construct strongly elevated the generation of specific IgG antibodies following intraperitoneal administration (44). We concluded that the Montanide M720-adjuvanted vaccine elicited higher levels of IFN-γ compared with the alum-adjuvanted one. Similar to our results, Mutiso et al. found that the Montanide M720-adjuvanted vaccine could induce more levels of IFN-γ in comparison with the alum-containing formula (45). It has been reported that Montanide M720 adjuvant is involved in the generation of $CD4^+$ T lymphocyte clones and mice immunization with Montanide M720 adjuvant augments the frequency of IFN-γ-producing $CD4^+$ T-cells (46). Other studies established that adjuvanticity of Montanide is due to its ability to induce cellular immunity, followed by IFN-γ production (47, 48). Our study has some limitations. The immune responses were assessed in mice without modeling hepatitis. Moreover, we did not monitor immune responses, specifically memory cells, for a long time to determine the protective effect of vaccination regimens against infection with hepatitis viruses.

CONCLUSION

Protein-based subunit vaccine platforms are promising strategies in immune response induction, especially against highly variable viruses, with the advantage of effectively stimulating the specific immune response. This current research demonstrated that the newly designed truncated VP1+HBsAg could trigger strong immune reactions in BALB/c mice, indicating its potential for further exploration as a bivalent vaccine targeting both HAV/HBV. For future work, we suggest the efficacy of the bivalent vaccine to be evaluated in transgenic, transfected, or humanized chimeric mice in order to support the complete cycle of virus infection. Furthermore, constructing built-in adjuvant bivalent vaccines is proposed for future studies in which adjuvants are incorporated within the vaccine construct, improving the immunogenicity of epitopes.

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