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Immune Response Profile Induced by Combined Alum and Glycyrrhizin Liposomes in Balb/c Mice Immunized with Ovalbumin

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Abstract

Objective: Saponin compounds, including Glycyrrhizin, can enhance immune responses. The current investigation was conducted to study the effects of alum and Glycyrrhizin Liposomes (GL) as adjuvants on the humoral and cellular responses of the immune system in mice immunized with ovalbumin (OVA).

Materials and Methods: Male Balb/c mice were immunized with OVA adjuvant with alum, GL, or alum-GL combination twice at two-week intervals. Two weeks after the last immunization, the specific immune responses against OVA were assessed.

Results: The adjuvant formulated with alum and GL induced a Th1 cytokine pattern against OVA, while alum alone induced a Th2 cytokine pattern. The combined adjuvant increased the potential of OVA to induce a delayed- type hypersensitivity reaction and IgG2a antibody titer compared to other groups ($p<0.05$). OVA-specific lymphocyte proliferation did not show significant differences among the three groups receiving adjuvant ($p=0.11$).

Conclusion: Unlike alum, the combination of alum and GL synergistically increased the cellular and humoral immune responses after immunization with an antigen and therefore had the ability to be used as an adjuvant to induce cellular immune responses.

Keywords: Adjuvant, alum, glycyrrhizin liposome, ovalbumin

Introduction

Effective immune responses to protect against intracellular infectious agents require the simultaneous cooperation of cellular and humoral immune responses (1-4). One of the simplest methods to protect against pathogenic microorganisms is to use their killed form along with adjuvants (1,2). However, alum, the most common adjuvant, is only capable of inducing humoral immune responses (3,4). Another approach to generating proper cellular immune responses against obligate or facultative intracellular pathogens is to use a strain of these microorganisms with sufficient immunogenicity and low virulence (1,4). Unfortunately, this form of microorganisms is not always available (1). Therefore, designing new types of adjuvants that can create a cellular immune response after using the killed form of pathogens is a logical way to enhance protection against such microorganisms (1). However, many adjuvants that have produced cellular immunity in animal models cannot be used in humans due to their toxicity or high cost (3). For instance, Freund's complete adjuvant is one of the common powerful adjuvants used to induce cellular immunity and Th1 responses (4,5). Nevertheless, its application is restricted to use only in laboratory animals due to its side effects and inducing reactions associated with severe pain (5). Therefore, the study and design of new adjuvants are still needed to induce cellular immune responses.

Saponins are a heterogeneous group of sterols and triterpene glycosides that have been isolated from a wide

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range of plants (6). It has been shown that saponins have the potential to stimulate the immune system of animals, and there has been great interest in using them as adjuvants (7). Glycyrrhizin or glycyrrhizic acid is a triterpenoid saponin extracted from the licorice plant, which possesses antitussive, wound healing, antiviral, and interferon production induction properties (7,8). The beneficial effect of glycyrrhizin tablets in the adjuvant treatment of purpura simplex Henoch-Schönlein has been reported in the past (9). However, using this agent led to challenges such as low solubility. One solution to overcome this problem is to introduce Glycyrrhizin into the liposome to increase its delivery (7).

Liposomes are bilayer vesicles that form spontaneously after the distribution of amphiphiles in aqueous environments (10). Phospholipids, such as phosphatidylcholine and phosphatidylglycerol, are among the amphiphiles, and other substances like cholesterol are often included in the formulation (9,10). Hydrophilic compounds can be snared inside the liposome, while lipophilic compounds are usually included in the liposome membrane (10). Liposomes have become popular due to their potential and actual use in targeted drug delivery. Liposomes are known for their potential and actual use in targeted drug delivery (11). In addition, liposomes seem to have many advantages such as low cost, high stability, and biodegradability along with the ability to stimulate humoral and cell-mediated immune responses (10,11).

Ovalbumin (OVA) makes up about 75% of the total protein in egg white. OVA is a non-toxic and inert compound that is used in immunological research as a model protein to evaluate specific immune responses to an antigen in different mammals such as mice (12). In the present study, the effect of the combined alum and glycyrrhizin liposome (GL) as a combined adjuvant on the humoral and cellular immune responses in reaction to OVA in Balb/c mice has been investigated.

Materials and Methods

Immunization

The study population comprised male Balb/c mice aged six to eight weeks, purchased from the animal center of the veterinary faculty of Urmia University, Iran. The main basis for the ethical considerations of animal research was the Helsinki Convention. This research was conducted after the evaluation and approval of the Ethics Committee of our faculty (Veterinary Ethics Committee of Urmia University (no: IR-UU-AEC-3/5, date: 14.06.2023). After one week of adaptation, the mice were allocated into five groups, each consisting of seven mice. The mice were immunized subcutaneously with OVA in a volume of 150 microliters, twice, with an interval of 14 days. Immunization protocol was done subcutaneously and on loose skin on the neck.

Immunization protocol for the OVA/GL/A group included a combination of alum (potassium aluminum sulfate) and GL was provided by mixing 50 µL of normal saline containing GL (1 mg/mL) with 50 µL of alum (A). Then 50 µL of the OVA solution in normal saline (2 mg/mL) was absorbed in 100 µL of the combined GL/A mixture.

To prepare the immunization protocol for the OVA/A group, OVA solution in normal saline (2 mg/mL) was absorbed in 50 µL of alum.

For the OVA/GL/group, OVA (2 mg/mL) was mixed with 50 µL of GL (1 mg/mL).

To provide the immunization protocol for the OVA group, a solution containing 2 mg of OVA in normal saline was prepared.

A subcutaneous injection of 150 µL of normal saline was used to challenge the control mice.

Preparation of Micelles Containing Glycyrrhizin

Liposomes were prepared using the dehydration/rehydration method (12). For this purpose, two phases, aqueous and organic, were prepared separately and mixed as follows (volume ratio of organic phase: aqueous=45:55) to obtain a uniform solution with the final volume.

The organic phase consisted of 40 mg of soy phosphatidylcholine and 10 mg of cholesterol, dissolved in a volume of 6.2 mL of tert-butyl alcohol at a temperature of 45°C.

The aqueous phase contained 5 mg of Glycyrrhizin and 120 mg of sucrose, solved in 1.2 mL of deionized water at 45°C. The resulting dilution was lyophilized, and a white powder was obtained and redispersed in 4.7 mL of deionized water at 65°C.

Liposome Encapsulation Efficiency

Briefly, one hundred microliters of liposome were added in a 10 mL centrifuge tube and mixed with 100 microliters of protamine solution (10 mg/mL). After 180 seconds, normal saline was added to the tube in a volume of 3 mL and centrifuged at 4000 rpm at room temperature for 30 minutes. After collecting the supernatant, 2 mL of it was used to measure the content of free Glycyrrhizin by vitriol-vanillin method (13). Glycyrrhizin is a saponin compound. The amount of saponin present in a sample can be determined by the simple and quick vanillin-vitriol method (14). In order to measure the amount of encapsulated Glycyrrhizin, the sediment remaining in the centrifuge tube was dissolved with 0.6 mL of Triton X-100 and 2.6 mL of physiological saline. Two milliliters of the solution were taken and the amount of Glycyrrhizin was measured by vitriol-vanillin method.

Finally, this formula was used to calculate the liposome encapsulation efficiency (EE):

EE % = (1 - Cf/Ct), Cf: the content of free Glycyrrhizin, Ct: the total content of Glycyrrhizin (13).

Assessment of Delayed Type Hypersensitivity (DTH)

Two weeks after the last challenge, 50 µL of OVA (1 mg/mL in equal volumes of PBS) was immunized subcutaneously into the plantar base of the left foot of five mice from each group. The negative control mice were only challenged with normal saline. A digital caliper was applied to measure the thickness of the foot pads. The amount of DTH was recorded based on this formula: [(thickness of right foot) - (thickness of left foot)] x 100/(thickness of left foot) (1).

Ex vivo Immunological Evaluations

Fourteen days after the last challenge, five mice from each group were subjected to deep anesthesia. After blood collection, the mice's serum was separated. The specific titer of IgG against OVA and the IgG2a/IgG1 ratio were then determined using the ELISA method (1).

The spleens of the Balb/c mice were aseptically dissected, shrieked, and passed via a 20 µm diameter wire mesh. A mononuclear cell suspension was separated by Ficoll-Hypaque density gradient centrifugation, and erythrocytes were deleted using ACK (Ammonium-Chloride-Potassium) lysis buffer. Isolated splenocytes (1×10^6 cells/mL in RPMI-1640 medium plus 10% fetal calf serum) were kept in 6-well plates and stimulated with 100 µg/mL OVA. After 72 hours, the culture supernatant of spleen cells was gathered and used to measure interleukin (IL)-4 and interferon gamma (IFN-γ) cytokines by ELISA method pursuant to the manufacturer's guidelines.

Moreover, isolated mononuclear cells were kept in 96-well flat-bottomed microplates (10^5 cells/100 µL/well) and were stimulated with 100 µg/mL OVA or the medium alone. After 72 hours, 25 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was mixed to each well and after four hours of incubation, the supernatant was removed. Then, 150 µL of dimethyl sulfoxide was pulsed to each well and the crystals were dissolved by rapid pipetting. The microplates were monitored at a wavelength of 570 nm with an ELISA reader. The splenocyte proliferation index was recorded pursuant to the ratio of the absorbance of the splenocytes stimulated with OVA to the absorbance of non-stimulated spleen cells (15).

Statistical Analysis

First, the normal distribution of the data was confirmed using the Kolmogorov-Smirnov test. Then, the data were analyzed using a one-way analysis of variance (ANOVA) using Tukey's post hoc test. A p-value of 0.05 or less was considered statistically significant. Data were reported as mean ± standard deviation.

Results

To evaluate the liposome encapsulation efficiency, three batches of GAL were prepared. The encapsulation rate was calculated as 75.27 ± 1.1%.

The mice immunized with OVA/GL/A exhibited the highest percentage of DTH reaction compared to other experimental groups (p=0.03). On average, a 3.11-fold increment in the violence of this reaction was recorded in mice immunized with OVA/GL/A, compared to the experimental group that received OVA (Figure 1). The percentage of DTH was higher in the OVA/A and OVA/GL groups in comparison with the DTH intensity in the OVA and control mice [(p=0.04), Figure 1]. The average increment in DTH intensity in mice immunized with OVA/A or OVA/GL was 1.92 times and 2.29 times higher than in the OVA-immunized mice, respectively. Data analysis indicated that there was no statistical difference in DTH reaction between the OVA/A and OVA/GL groups [(p=0.09), Figure 1].

The proliferation intensities of splenic lymphocytes in the OVA/GL/A, OVA/GL, and OVA/A groups were significantly higher than in the OVA-immunized mice or control group (p=0.02). However, there was no significant diversity among the last three groups [(p=0.11), Figure 2a].

According to Figure 2b, the highest production of IFN-γ by OVA-stimulated splenocytes was recorded in

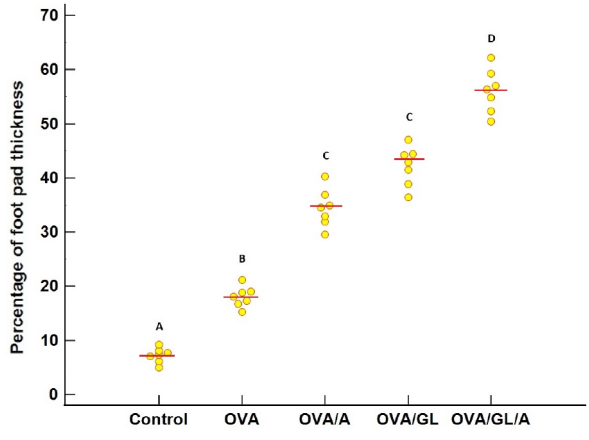

Figure 1. Effects of immunization protocols on DTH reaction. The male Balb/c mice were subcutaneously challenged twice at two-week intervals. Two weeks after the last immunization, 50 µL of OVA (1 mg/mL) was subcutaneously injected into the left hind paw of 5 mice from each experimental group. The negative control mice received the same volume of normal saline. The footpad thickness was monitored by a digital caliper after 48 h. Each point is the finding from an individual mouse. The line drawn across each group indicates the mean (Different letters indicate different statistical difference at the level of p<0.05).
OVA: Ovalbumin, DTH: Delayed Type Hypersensitivity

the supernatant of spleen cells from mice that received OVA/GL/A. Immunized mice with the combined adjuvant exhibited a 25.9-fold enhancement in IFN-γ production in comparison with the group challenged with OVA alone [(p=0.04), Figure 2b]. The level of IFN-γ was mounted in the spleen cell soup of mice challenged with OVA/GL compared to the spleen cells of mice that were challenged with OVA or OVA/A. The IFN-γ production by splenocytes of mice that were immunized with OVA/A was statistically higher than that of mice in the OVA-immunized group [(p=0.03), Figure 2b].

The production of IL-4 in the supernatant of splenocytes of mice treated with OVA/A was significantly higher compared to other groups [(p=0.03), Figure 2c]. IL-4 level was premier in the supernatant of spleen cells isolated from mice immunized with OVA/GL, compared to spleen cells of mice immunized with OVA/GL/A [(p=0.01), Figure 2c]. It should be noted that ELISA did not detect the level of IL-4 and IFN-γ in the control group.

Statistically, the groups that received OVA/GL/A or OVA/A had the highest specific IgG titers against OVA compared to the other groups [(p=0.03), Figure 3a]. The antibody titer in the OVA/GL group was higher in comparison with mice challenged with OVA or control groups. However, it was significantly lower than the groups immunized with OVA/A and the mice challenged with OVA/GL/A [(p=0.01), Figure 3a]. As illustrated in Figure 3b, the IgG2a/IgG1 ratio was significantly mounted in mice immunized with OVA/GL/A compared to animals immunized with other protocols. The ratio of IgG2a/IgG1 in OVA/GL group mice was significantly higher compared to OVA/A, OVA, and control mice [(p=0.04), Figure 3b]. Significantly, the ratio of IgG2a/IgG1 in mice immunized with OVA/A was lower than that in animals that received only OVA [(p=0.02), Figure 3b].

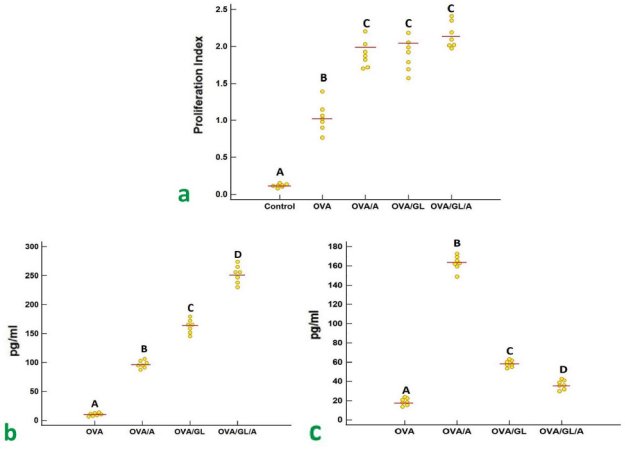

Figure 2. Effects of immunization protocols on OVA-specific splenocyte proliferation and secretion of IFN-γ and IL-4. Two weeks after the last immunization, the splenocyte suspension was cultured and pulsed with OVA for 72 h to analyze the lymphocyte proliferation potential (a), and secretion of IFN-γ (b) and IL-4 (c) by the ELISA method. It should be noted that the level of IFN-γ and IL-4 in the control group was not detectable by ELISA. The footpad thickness was monitored by a digital caliper after 48 h. Each point is the finding from an individual mouse. The line drawn across each group indicates the mean (Different letters signify a statistical difference at the level of $p<0.05$).
OVA: Ovalbumin, IFN-γ: Interferon gamma, IL: Interleukin

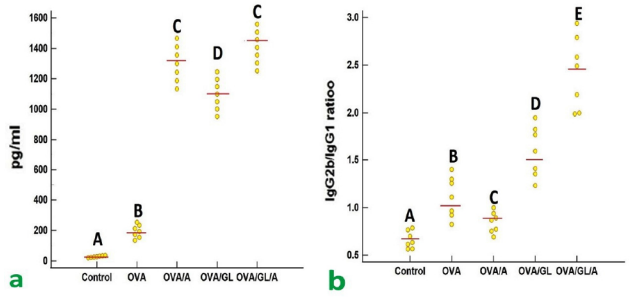

Figure 3. Assessment of immunization on antibody responses. The blood samples were taken from the mice two weeks after the last immunization, and their sera were gathered. The specific IgG titer (a) and their isotypes against OVA (b) were evaluated by the ELISA method. The footpad thickness was monitored by a digital caliper after 48 h. Each point is the finding from an individual mouse. The line drawn across each group indicates the mean (Different letters dealer a statistical difference at the level of $p<0.05$).
OVA: Ovalbumin

Discussion

The rationale for using adjuvants includes the slow and continuous release of antigens and the induction of the co-stimulatory molecules, like B7-1 (CD80) and B7-2 (CD86), by antigen-presenting cells (APCs), consisting of dendritic cells, macrophages, and B lymphocytes, as well as orientation of the release of cytokines that polarize the immune system (16-18). The response of APCs is important for the efficacy of vaccines. The rationale of combined medication to manage complex diseases is according to the use of two or more medications with varied therapeutic mechanisms. Similarly, the combined adjuvants to gain synergy and obtain a more appropriate result need identical reasoning (17,18). Since the beginning of the 20th century, potassium aluminum sulfate or alum has been recognized as the most popular adjuvant (1). In addition to the property of storage and slow release of antigen, alum induces cytokines and chemokines through the induction of inflammation by tissue macrophages, which ultimately leads to the accumulation, maturation, and migration of APCs after harvesting. Altogether, alum induces a Th2 response (19).

In the past, it has been shown that dendritic cells treated with glycyrrhizin were able to influence the polarization of T-cells towards the Th1 subset and thus generate cellular

immune responses (20). In addition, glycyrrhizin can block prostaglandin-E2 synthesis by blocking cyclooxygenase-2, which leads to a simultaneous increase in nitric oxide production by increasing *iNOS2* gene expression in macrophages infected with Leishmania (21). In other words, glycyrrhizin leads to the strengthening of the inflammatory potential of macrophages. Glycyrrhizin was able to block the expression of Th2, IL-10, and TGF-β from the spleen cells of Leishmania-infected mice (22). Glycyrrhizin has also been reported to promote the maturation of dendritic cells in mice. This adjuvant-like potential may have a beneficial therapeutic effect due to the increased expression of B7-1, B7-2, CD83, CD40, and major histocompatibility complex type II (MHC II) (23). Due to the low solubility and side effects of glycyrrhizin, it is logical to introduce this compound in a liposomal form. Liposomes lead to the efficient delivery of glycyrrhizin to APCs (7).

The results of the present study have suggested that GL enhance cellular immune responses compared to alum. More importantly, the combination of GL and alum liposomes promoted stronger IFN-γ (the main cytokine of Th1 responses) and simultaneously enhanced humoral and cellular immunity. Previous research has shown that immune responses to the *S. typhimurium* vaccine can be potentiated through the use of metoclopramide-alum, propranolol-alum, or alum-naloxone mixtures as vaccine adjuvants (1,24,25). Unlike an immune-stimulating compound, an adjuvant must be given in entire combination with an antigen to promote its adjuvant benefit (1). In the current investigation, GL or alum were also administered in a mixture with OVA.

Previous results showed that Glycyrrhizinliposomes significantly increased the antibody titer of IgG and IgM classes in chickens immunized with the Newcastle vaccine (7). Alum is also one of the popular adjuvants that augments antibody development pursuant to the use of the killed preparation of microbes (26). Our results showed that both alum adjuvants and GL were favored in mounting neutralizing antibodies against OVA. In addition, the combined GL and alum have a synergistic benefit in the generation of neutralizing antibodies against the OVA antigen.

T lymphocyte responses can polarize in different directions and functions according to the type of cytokines presented by APCs. IFN-γ is known as the main cytokine of Th1 responses. Also, IL-4 is considered the main Th2 cytokine (27). The microenvironment created by adjuvants promotes the polarization of the responses of the immune system. The potential of alum in driving immune responses towards Th2 is well-known. As mentioned, contrary to Alum, dendritic cells treated with Glycyrrhizin were able to influence the differentiation of T-cells towards the Th1 subset (20). It has been reported that adjuvant treatment of Henoch-Schönlein's purpura simplex with glycyrrhizin was superior in improving immune function, especially in improving serum T lymphocyte subsets compared to the control group (10). Following the use of glycyrrhizin tablets, patients with Henoch-Schönlein's purpura simplex had a markedly increased $CD4^+$ T-cell count, while their $CD8^+$ T-cell count was significantly lower than in patients who received conventional treatment (9). Our findings suggest that *ex vivo* challenge of spleen cells gathered from mice challenged with OVA and alum resulted in higher IL-4 production compared to other groups. On the contrary, *ex vivo* stimulation of spleen cells of mice challenged with OVA and combined GL and alum resulted in higher production of IFN-γ compared to other groups. The second level of IFN-γ was observed in the supernatant of splenocytes isolated from mice immunized with OVA and GL. Based on our results, the level of IL-4 in the supernatant of spleen cells isolated from mice immunized with OVA/A was superior compared to supernatant of spleen cells isolated from mice immunized with OVA/GL. Interestingly, it has been shown that treatment with glycyrrhizin decreased IL-4 and IgE levels in mice that induced allergic rhinitis by subcutaneous injection of OVA and alum followed by intranasal administration of OVA. Also, in mice with allergic rhinitis and treated with glycyrrhizin, the IL-12 (IFN-γ inducing factor) was increased compared to untreated mice with allergic rhinitis (28).

T lymphocytes are responsible for the generation of high-titer IgG antibodies (29). Both Th1 and Th2 responses are effective in creating an effective anti-antibody response. However, Th1 responses promote the production of antibody isotypes with stronger complement activation and opsonization potential than Th2 responses (1,27). In mice, IFN-γ supports an IgG2a isotype switch, whereas IL-4 supports an IgG1 isotype switch (27). In our study, the highest ratio of IgG2a to IgG1 antibodies was recorded in the mice that were immunized with OVA mixed with the combined adjuvant. Based on attained data, animals immunized with OVA and alum had less IgG2a/IgG1 ratios compared to the mice immunized with OVA and GL. These findings were completely consistent with the data recorded from the analysis of IL-4 and IFN-γ production in supernatants of splenocytes.

Evaluation of DTH responses is one of the most popular methods to monitor the responses of the cellular arm of the immunity system against a specific antigen. Th1 and macrophages are the main players in the DTH reaction (30). The findings of this assay were consistent with the results attained from the evaluation of cytokine analysis in the supernatant of splenocytes, and the foremost

outcomes were considered in mice immunized with OVA and combined adjuvants. The intensity of OVA-specific lymphocyte proliferation was another assay used in the current investigation to assess the cellular arm of immunity. Hereon, the data also showed the synergy between GL and alum in augmenting the immune responses induced by the OVA vaccine. The evaluation of lymphocyte proliferation intensity did not show any statistical difference between the groups immunized with OVA/A, OVA/GA, and OVA/GA/A. Furthermore, the findings related to the production ratio of cytokines as well as the severity of DTH showed a significant difference between the immunized mice. These findings represent the effect of the combined adjuvant on polarizing immune responses towards cellular immune responses, instead of simple immune stimulation.

Conclusion

Overall, the use of GL and alum as a combined adjuvant with OVA antigen can induce both humoral and cellular immunity while polarizing the responses of the immune system towards Th1 immunity. As a result, this combined adjuvant has the potential to offer new and safe tools for generating profound cellular immune responses against various microbes and cancers. Moreover, the study's findings suggest that administering GL, even without alum, in conjunction with an antigen can enhance cellular immunity.

Ethics

Ethics Committee Approval: The main basis for the ethical considerations of animal research was the Helsinki Convention. This research was conducted after the evaluation and approval of the Ethics Committee of our faculty (Veterinary Ethics Committee of Urmia University (no: IR-UU-AEC-3/5, date: 14.06.2023).

Informed Consent: Not necessary.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.M.A.F., Design: S.M.A.F., Data Collection or Processing: S.T., Analysis or Interpretation: S.T., S.M.A.F., R.Y., Literature Search: S.T., R.Y., Writing: S.M.A.F.

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