CLINICS

BASIC RESEARCH

The preventive effects of natural adjuvants, G2 and G2F on tracheal responsiveness and serum IL-4 and IFN-γ (th1/th2 balance) in sensitized guinea pigs

Mohammad Hossein Boskabady,[I*] Ali Neamati,[II] Saleh Mohaghegh Hazrati,[III] Mohammad Reza Khakzad,[IV] Shakeeb Hassan Moosavi,[V] Zahra Gholamnezhad[I]

[I] Mashhad University of Medical Sciences, School of Medicine, Neurogenic Inflammation Research Centre and Department of Physiology, Mashhad, Islamic Republic of Iran. [II] Islamic Azad University, Department of Biology, Mashhad Branch, Islamic Republic of Iran. [III] Tehran University of Medical Sciences and Dr. Mohaghegh's Foundation Research on Industrial Biotechnology, Tehran, Islamic Republic of Iran. [IV] Islamic Azad University, Mashhad Branch, Mashhad, Iran Zakaria Research Center, Mashhad, Iran. [V] Faculty of Medicine, NHLI, Imperial College, London/UK.

OBJECTIVE: The effects of natural adjuvants on lung inflammation and tracheal responsiveness were examined in sensitized guinea pigs.

METHODS: The responses of guinea pig tracheal chains and the serum levels of interleukin-4 and interferon-gamma were examined in control pigs and three other groups of guinea pigs: the sensitized group and two other sensitized groups treated with either adjuvant G2 or adjuvant G2F (n = 7 for each group). Sensitization of the animals was achieved by injection and inhalation of ovalbumin.

RESULTS: The results showed that sensitized animals had increased tracheal responsiveness and increased serum levels of interleukin-4 and interferon-gamma compared to controls ($p<0.05$ to $p<0.001$). Treatments with either G2 or G2F prevented the increase in tracheal responsiveness and serum interleukin-4 ($p<0.01$ to $p<0.001$). However, the serum levels of interferon-gamma and the interleukin-4-to-interferon-gamma ratio was increased in the treated groups ($p<0.001$ for all cases).

CONCLUSIONS: These results indicate important preventive effects of two natural adjuvants, particularly G2, on the changes in tracheal responsiveness, serum cytokines and the interleukin-4-to-interferon-gamma ratio (T helper 1/T helper 2 balance) in sensitized guinea pigs.

KEYWORDS: Natural Adjuvant; Asthma; Tracheal Responsiveness; Cytokines; Sensitized Animals.

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E-mail: boskabadymh@mums.ac.ir; mhboskabady@hotmail.com

*corresponding author

Tel.: 9 1 7794-8564

INTRODUCTION

The hallmark of asthma is airway hyper-responsiveness (AHR) to many stimuli (1), and it is believed to be due to airway inflammation (2). In addition, the T helper 2 system is activated in asthma, and its mediators cause many features of asthma, such as airway inflammation, mucus secretion and airway hyper-responsiveness (3). The T helper 1 system has been shown to inhibit T helper 2 responses. Therefore, one goal of asthma therapy should be focused on increasing the activity of the T helper 1 system by means of natural immunomodulators (4).

G2 is buffalo spleen extract. Theories of alternative medicine have suggested that the consumption of spleen extract supports the immune system (5,6). The effects of calf spleen extract, including inhibiting lymphocyte proliferation in a tissue-specific, species-nonspecific, nontoxic and reversible manner (7) and stimulating polymorphonuclear leukocyte activities (8), have been confirmed. Phosphodiesterase enzymes, nucleotidyltransferase activity (9) and a factor that inhibits DNA synthesis found in lymphocytes from the lymph nodes and thymus of guinea pigs and rats have also been isolated from the calf spleen (10).

G2F adjuvant is concentrated G2 adjuvant in sesame seed oil. Sesame seed has been used to make oil since ancient times. The earliest sesame findings date back to 3000 years BC in the history of the Middle East. Ancient athletes used it to enhance their power and physical fitness. The pure oil of sesame seeds and its components have been used as

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adjuvants for various purposes; this oil has been reported to increase vaccine potency (11), enhance cellular immunity as an adjuvant (12) and improve natural killer cell activity (13), but it does not affect antibody production (14). Moreover, sesame oil has antioxidant activity, inhibits lipid peroxidation and can be used as a drug delivery system (15,16).

The effect of the G2 adjuvant on breast cancer (17) and its stimulatory effects on Th1 cells have also been observed (18). The therapeutic effects of the G2 adjuvant on different illnesses such as allergies and asthma, have also been suggested (19). Therefore, the therapeutic effects of this adjuvant on different disorders and its mechanisms of action should be studied.

The safety of this adjuvant was demonstrated by daily injection of eight different doses of G2, including 1, 2.5, 5, 10, 20, 40, 80 and 160 μg, in mice (i.p.) for 18 weeks (n = 20 for each dose). Only a few deaths occurred, mostly among the animals receiving high doses of adjuvant (18 of the total of 160 animals). There was no relationship between the number of dead animals and the administered doses.

In our earlier study, the separate and combined preventive effects of G2 and PC (another adjuvant of bacterial origin) on sensitized guinea pigs were demonstrated (20). Therefore, in the present study, we examined the effects of the G2 and G2F adjuvants on the tracheal responsiveness and blood cytokine levels of sensitized guinea pigs.

MATERIALS AND METHODS

Animal sensitization and different groups

Guinea pigs were sensitized to ovalbumin (OA) (Sigma Chemical Ltd UK) by injecting 100 mg i.p. and 100 mg s.c. on day 1 and a further 10 mg i.p. on day 8. From day 14, the sensitized animals were exposed to an aerosol of 4% OA for 18 ± 1 days for 4 min daily (21-23). The aerosol was administered in a closed chamber (dimensions: 30×20×20 cm). Control animals were treated similarly, but saline was used instead of OA solution. The study was approved by the ethical committee of the Mashhad University of Medical Sciences.

A total of 28 adult Dunkin-Hartley guinea pigs (550 to 700 g, purchased from Razi Animal Institute, Mashhad, Iran) and were divided into four groups of 7 animals each, with a similar male-to-female ratio. All of the female animals were studied on the same days of their estrous cycles. The four groups were created as follows: 1) C: non-sensitized, control group; 2) S: animals sensitized to ovalbumin (an animal model of asthma) that received normal saline, 0.5 ml i.p. twice a week for 4 weeks; 3) S+G2: sensitized animals treated with adjuvant G2, 0.4 ml i.p. twice per week for 4 weeks; and 4) S+G2F: sensitized animals treated with adjuvant G2F, 0.1 ml i.p. twice per week for 4 weeks. The doses of adjuvants used were according to previous studies (19,20).

The animals were group-housed in individual cages in climate-controlled animal quarters and were given water and food *ad libitum*, while a 12-h on/12-h off light cycle was maintained.

Studied adjuvants

The G2 adjuvant is a buffalo spleen lipid that is registered as a patent in the Iranian Patent Office as follows: Immune System Activator Vaccine (Innovation Register No.: 36679, October 28, 2006). Briefly, spleens were crushed and diluted in alcohol for a few days, before being centrifuged at 800×g for 30 mins. The supernatant was dried to obtain a concentration of 20 μg/ml. The G2 adjuvant's components included different types of lipids, glucose, cholesterol and triglycerides.

For the G2F preparation, crude sesame seed oil from the Esfahan state in central Iran was filtered with 0.5-μm filter paper to remove particles. The oil was then centrifuged at 10000 rpm at room temperature. The supernatant was filtered with 0.22-μm pure-sized filters under vacuum filtration. A total of 300 μg/ml G2 was added to the resulting preparation; the mixture was called G2F and divided into 5-ml aliquots in glass vials under sterile conditions.

Tissue preparations

The guinea pigs were sacrificed by cervical dislocation and the trachea was removed. Each trachea was cut into 10 rings (each containing 2-3 cartilaginous rings) and the rings were cut open opposite the trachealis muscle and were sutured together to form a tracheal chain (24,25).

The tissue was suspended in a 10-ml organ bath (organ bath 61300, BioScience Palmer-Washington, Sheerness, Kent, U.K.) containing Krebs-Henseliet solution with the following composition (mM): NaCl 120, $NaHCO_3$ 25, $MgSO_4$ 0.5, KH_2PO_4 1.2, KCl 4.72, $CaCl_2$ 2.5 and dextrose 11. The Krebs solution was maintained at 37℃ and was aerated with 95% O_2 and 5% CO_2. The tissue was suspended under isotonic tension of 1 g and was allowed to equilibrate for at least 1 h, while it was washed with Krebs solution every 15 min.

Assessment of the tracheal response to methacholine

A cumulative log concentration-response curve of methacholine hydrochloride (Sigma Chemical Ltd., Poole, Dorset, U.K.), which induced contraction of the tracheal chain, was obtained in each experiment. Different concentrations of methacholine were added every 2 min: 0.1, 0.5, 1, 5, 10, 50 μM, 0.1, 0.5, 1 and 5 mM. The contraction elicited by each concentration was recorded at the end of 2 min and the effects plateaued in all of the experiments. The percentage of contraction of the tracheal smooth muscle due to each concentration of methacholine, in proportion to the maximum contraction obtained by its final concentration, was plotted against the log concentration of methacholine to obtain the curve.

The contractility response to 10 μM methacholine, which is the concentration that caused approximately 60-70% of maximum response, was also measured as the magnitude of contraction in milligrams. The effective concentration of methacholine causing 50% of the maximum response (EC_{50}) was also determined from each methacholine response curve.

Measurement of IL-4 and IFN-γ in serum

Five milliliters of peripheral blood were obtained immediately after sacrificing the animals and were kept at room temperature for 1 h. The samples were then centrifuged at 2500 rpm at 4℃ for 5 min. The supernatant was collected and immediately stored at -20℃ until the time of analysis. The serum levels of IL-4 and IFN-γ were measured using an interleukin-4 (IL-4) and interferon-γ (IFN-γ) ELISAA kit (Antibodies-online Inc., Atlanta, GA, USA), with a minimum detection limit of 1 pg/ml.

Statistical analysis

The data are reported as means ± SEM. The ratio of IL-4 to IFN-γ was calculated. According to the Kolmogorov-Smirnov test, these data had a normal distribution. The data of the four groups of animals were compared using ANOVA. Significance was accepted at $p<0.05$.

RESULTS

Tracheal response to methacholine

The cumulative log concentration response curve obtained in the sensitized (S) group showed a leftward shift, compared to the curve for the control group. However, the curves obtained in the S+G2 and S+G2F groups showed rightward shifts compared to the S group so that the curve obtained in the S+G2 and S+G2F groups was also shifted slightly to the left, compared to the curve of the control group (Figure 1).

The mean EC_{50} value in the tracheal chains of the S animals (0.59 ± 0.11 μM) was significantly lower than the EC_{50} value in the control group (4.10 ± 0.62 μM, $p<0.001$) (Figure 2a). However, the EC_{50} value in the S+G2 (4.43 ± 0.88 μM) and S+G2F (4.21 ± 0.44 μM) groups showed significant improvement, compared to that in the S group ($p<0.001$ for both treated groups, Figure 2a).

Contractility response to methacholine

The mean value of the contractility response to methacholine in the tracheal chains of the S animals (217.14 ± 14.90 mg) was significantly greater than that in the control group (109.29 ± 19.00 mg, $p<0.001$) (Figure 2b). However, the maximum contractility response in the S+G2 (88.00 ± 8.10 mg) and S+G2F (135.86 ± 7.16 mg) groups showed significant improvement, compared to that of the S group; these values were statistically significant for the S+G2 and S+G2F groups ($p<0.001$ and $p<0.01$ for S+G2 and S+G2F, respectively, Figure 2b).

Levels of IL-4 and IFN-γ in serum

There was a significant increase in the levels of IL-4 and IFN-γ in the serum of the S animals, compared to those in the controls ($p<0.05$ for both cases). Both treatment groups were associated with a small but significant reduction in IL-4 ($p<0.01$ for both cases) and a substantial, highly significant increase in IFN-γ ($p<0.001$ for both cases, Figure 3a and b). The ratio of IFN-γ to IL-4 did not change in the sensitized group, but it was significantly increased in both treated groups ($p<0.001$ for both cases, Figure 3c).

Differences in the different parameters between the two groups of animals treated with the adjuvants

There were no significant differences in the EC_{50} value, contractility response to methacholine, serum levels of IL-4 and IFN-γ or the IL-4-to-IFN-γ ratio between the S+G2F and S+G2 groups (Figures 1-3).

DISCUSSION

Increased tracheal response to methacholine and the maximum tracheal response to methacholine in the sensitized guinea pigs compared to those of the control animals were seen in the present study. Serum interleukins (INF-γ and IL-4) were also significantly increased in the sensitized animals, compared to the control group; these results were very similar to the results of our previous studies, using a similar method of sensitization (26-28). Increased tracheal responsiveness to methacholine and serum cytokine levels constitute further confirmation of sensitization (28,29).

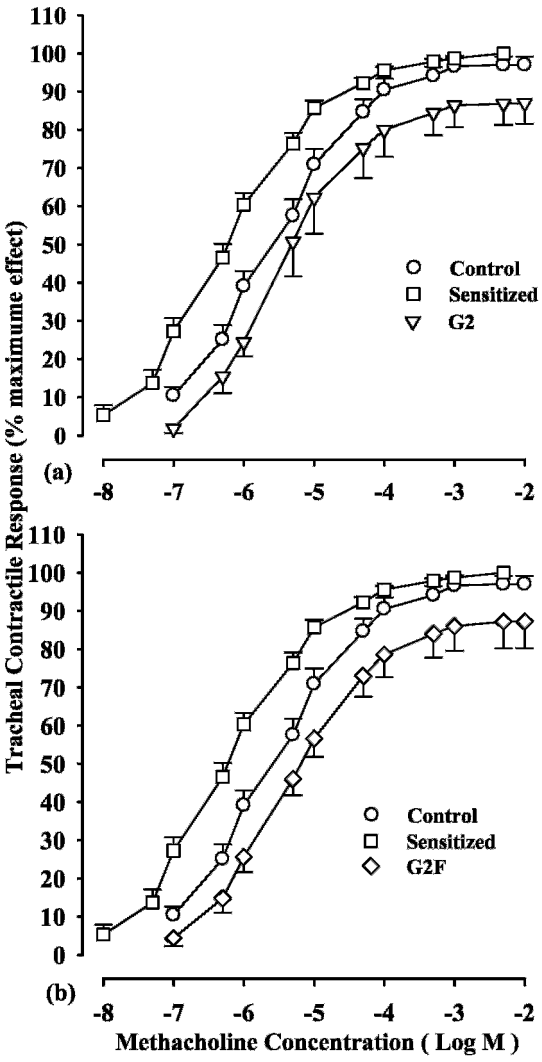

Figure 1 - Cumulative log concentration-response curves of methacholine-induced contraction of the isolated trachea in the control (C), sensitized (S), S treated with adjuvant G2 (S+G2) and S treated with adjuvant G2F (S+G2F) guinea pigs (for each group, n = 7).

Treatment of the S animals with G2 or G2F adjuvants prevented the increase in tracheal responsiveness to methacholine and serum levels of IL-4, but it caused further increases in serum INF-γ levels and increased the INF-γ-to-IL-4 ratio. There were no significant differences in the EC_{50} value, contractility response to methacholine, serum levels of IL-4 and IFN-γ or the IL-4-to-IFN-γ ratio between the S+G2F and S+G2 groups.

The preventive effects of the studied adjuvants on increased tracheal responsiveness, IL-4 and IFN-γ levels and the IL-4-to-IFN-γ ratio changes in sensitized guinea pigs indicated that the adjuvants had anti-inflammatory properties.

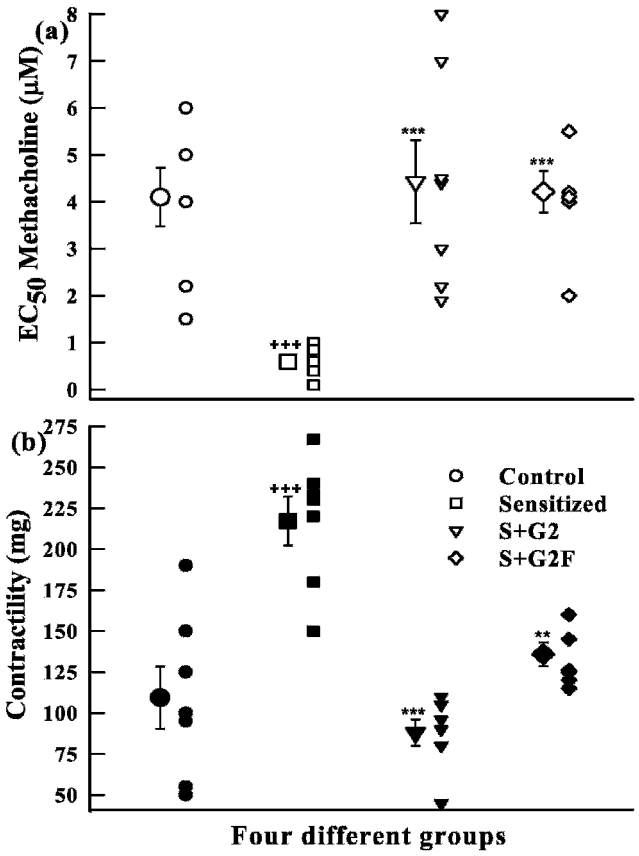

Figure 2 - Individual values and means ± SEMs (large symbols with bars) of the tracheal response to methacholine (EC_{50}) (a) and maximum contractility (b) in the four groups of guinea pigs (n = 7): control (C), sensitized (S), S treated with adjuvant G2 (S+G2) and S treated with adjuvant G2F (S+G2F). Significant differences between the control and sensitized groups: +++; $p<0.001$. Significant differences between the G2 and G2F groups *vs* the S group: **; $p<0.01$, ***; $p<0.001$. There was no significant difference in the EC_{50} or maximum contractility between the S+G2 and S+G2F groups.

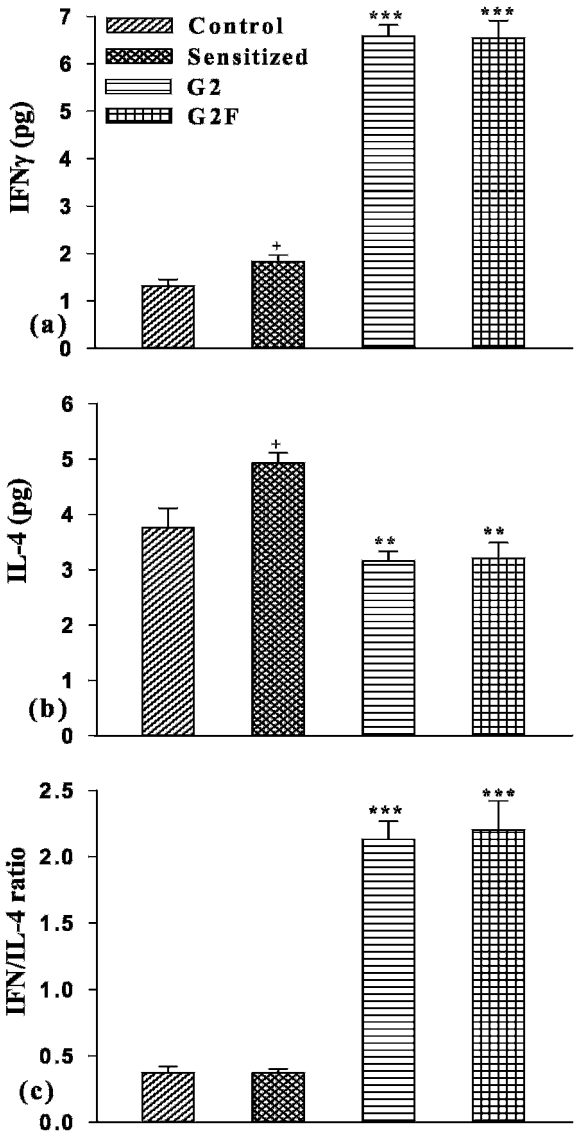

Figure 3 - Serum levels of IFN-γ (a), IL-4 (b) and IFN-γ-to-IL-4 ratio (c) in control, sensitized (S), S treated with adjuvant G2 (S+G2) and S treated with adjuvant G2F (S+G2F) guinea pigs (for each group, n = 7). Significant differences between the control and sensitized group: +; $p<0.05$. Significant differences between the S+G2 and S+G2F groups *vs* the S group: **; $p<0.01$, ***; $p<0.001$. There was no significant difference between the S+G2 and S+G2F groups.

The possible mechanism of action of these adjuvants is related to the regulation of the Th1 and Th2 balance. Th2 cells increase the activity of macrophages and regulate the pro-inflammatory response through the production of IL-4 and IL-10 but not of IFN-γ or IL-2, whereas Th1 cells inhibit the activity of macrophages directly or indirectly by inhibiting Th2 activity and thus regulating the anti-inflammatory response through interleukin 2 (IL-2) and interferon-γ (IFN-γ) (30). The preventive effects of the studied adjuvant on IL-4, IFN-γ and the IFN-γ/IL-4 ratio observed in the present study suggested the effect of adjuvants on the Th1/Th2 balance. The stimulatory effects of these adjuvants on Th1 cells (18) and the effects of calf spleen extract on the immune system (7-10) were demonstrated in previous studies that supported the findings of the present study.

Oxidative stress plays an important role in asthmatic airway inflammation and might be a useful therapy for bronchial asthma (31,32). Therefore, the antioxidant properties of the adjuvants might constitute another mechanism for their effects. In fact, the preventive effects of antioxidants in patients with asthma have been controversial (33-35). The effects of vitamin C and the extract of *Nigella sativa*, both of which have anti-oxidant properties against the increased tracheal responsiveness of sensitized guinea pigs using a similar method of sensitization, have been shown (36,37). Therefore, the antioxidant effects of these adjuvants should be examined in future studies. The preventive effects of the G2 and G2F adjuvants on lung inflammation (total and differential WBC counts) in sensitized guinea pigs were also demonstrated (38).

Sesame seed oil increases cellular immunity (11) and natural killer cell activity (12) and it has antioxidant activity (15,16). Therefore, the protective effects of extract of sesame seed could be due to its effects on immunity mechanisms or its antioxidant properties. Further studies are needed to

determine its effects on the regulation of Th1/Th2 balance and to measure directly the antioxidant properties of the adjuvants used in the current study.

The administered dose of the G2F form of the adjuvant was higher than that of the G2 adjuvant (30 μg *vs* 8 μg). In addition, the extract of sesame seed present in the G2F adjuvant demonstrated cellular immunity and antioxidant properties. However, the effects of the G2 adjuvant in the prevention of increased tracheal responsiveness (reduction of EC_{50}), contractility and eosinophil and neutrophil infiltration tended to be greater (although non-significantly) than those of G2F, and the G2 adjuvant's effects on total WBC and basophil counts were significantly greater than those of the G2F adjuvant. The mechanism of these discrepancies is currently unclear. One possible mechanism could be a non-specific adverse effect of a higher concentration of the adjuvant. However, the mechanism(s) of this discrepancy and possible adverse effects should be examined in further studies.

The results of the present study might suggest a preventive effect of the studied adjuvant on asthma by the regulation of the Th1/Th2 balance and/or anti-inflammatory effects. However, the other possible factors that influence these effects, including the dose of administration in asthmatic patients, the duration of effective treatment and tolerance by the patients, should be examined in further studies. In addition, the effects of different doses of the G2 and G2F adjuvants should be studied in sensitized animals before beginning human studies, to determine the optimal effective doses.

Regarding possible lymphocyte migration into different lung compartments from the spleen and its influence on lung immune response, it has been well documented that lymphocyte migration into the bronchoalveolar space after local challenge seems to be triggered by the inflamed lung and not by migrating cells from the spleen. Furthermore, the spleen is not the main source of lymphocytes in pulmonary immune reactions or cellular immune reactions (39). Increases in total WBC and in eosinophil, neutrophil and basophil counts in lung lavage of sensitized guinea pigs were also shown in our previous study (38). The effects of natural adjuvants on IL-4 and IFN-γ in lung lavage of a mouse model of experimental asthma also showed results that were very similar to those of the present study regarding both the quantity and effects of adjuvants (40), supporting the results of the present study. In addition, the effects of adjuvants on tracheal responsiveness shown in the present study, as well as their effects on total and differential WBC (38) and lung pathological changes (40), in sensitized animals confirmed their effects on lung inflammation in asthma.

In addition, the results of our recent study showed that, in cultured splenic lymphocytes from the control group, the levels of IFN-γ and IL-4 and the IFN-γ-to-IL-4 ratios were 3.91 ± 1.23, 2.82 ± 0.92 and 1.37 ± 1.07, respectively. In splenic lymphocytes that were stimulated with PHA, these values were 7.54 ± 2.15, 8.21 ± 1.28 and 0.83 ± 0.47, respectively, and in stimulated lymphocytes that were treated with the extract of *Nigella sativa*, these values were 13.82 ± 2.31, 4.06 ± 0.83 and 3.29 ± 0.86, respectively (unpublished data). In fact, the results with the extract of *Nigella sativa* (41) were very similar to those in the present study with the natural adjuvants. Therefore, these results also support the effects of these adjuvants on the serum levels of IFNγ and IL-4 and the IFN-γ-to-IL-4 ratio in the sensitized guinea pigs.

In conclusion, the results of the present study indicated the preventive effects of the G2 and G2F natural adjuvants on tracheal responsiveness, changes in serum cytokine levels and the IFN-γ/IL-4 ratio (Th1/Th2 balance) in sensitized guinea pigs.

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■ AUTHOR CONTRIBUTIONS

Boskabady MH designed the study, supervised the experiments, performed the statistical analysis and prepared the manuscript. Neamati A performed the experiments and was involved in the manuscript preparation. Khakzad MR performed the measurements of cytokine levels. Mohaghegh Hazrati S provided the adjuvants and assisted in the study design. Moosavi SH assisted in the manuscript preparation and statistical analysis.

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