Peptide ILE-GLU-TRP (Stemokin) Potential Adjuvant Stimulating a Balanced Immune Response

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Abstract
Vaccines are widely used worldwide to prevent and protect from various infections. A variety of modern approaches to developing prophylactic and therapeutic vaccines is growing. In almost all cases, adjuvants are necessary to obtain an effective immune response. This work investigated the possibility of using the pharmaceutical peptide drug Stemokin as an adjuvant stimulating a balanced Th1/Th2 response. A study was conducted to compare the activity of Stemokin versus the approved adjuvant Alhydrogel in a murine vaccination model with the approved VAXIGRIP® vaccine. The first proof-of-concept experimental study shows that the peptide Ile-Glu-Trp has the adjuvant vaccine properties and anti-HA IgG2a enhancing response, revealing a Th1-favoring balanced Th1/Th2 immunomodulation.

Keywords Adjuvants · Peptide pharmaceutical · Balanced Th1/Th2 immune response · Hematopoiesis · Cytokines

Introduction
The pandemic situation with Covid-19 in the world has boosted the development and global use of the various prophylactic vaccines. Modern approaches to the creation of effective and selective vaccines are developing in multiple directions - from the improvement of the manufacturing methods of traditional inactivated vaccines (Sinovac) (Zhao et al. 2020; Sadoff et al. 2021), vector-bearing vaccines (J&J, Sputnik) (Logunov et al. 2020), and mRNA-based matrix (Pfizer) (Walsh, E. et al. 2020), (Modern) (Jackson, L. A. et al. 2020) technology vaccines. On the other hand, the COVID-19 is spreading worldwide and infecting many people due to inadequate prophylactic measures. Therefore, the demand for Vaccines is overwhelming. Furthermore, the Vaccine must induce immunity quickly with the minimum amount of the antigen required. Thus, selecting an effective adjuvant becomes vital for developing an efficient Vaccine.

In the unprimed immune system, the invaded antigens are typically poor as a stimulant of the specific immune response, especially in vaccines where the immunizing antigen is an isolated individual DNA, RNA, and protein molecule or a synthetic polypeptide.

The organism’s harmful action induced by the invaded antigen stimulates an immediate reaction to generate strong protection. In response to the invasion, T lymphocytes, as a significant source of protective cells, start to create cytokines. There are two main subsets of T lymphocytes, distinguished by cell surface molecules known as CD4+. A different pattern of cytokine release defines CD4+ helper cell subgroups. The Th1 subset produces a cytokine profile to induce cell-mediated immunity, and the Th2 subgroup makes a cytokine profile to induce antibody synthesis. Both subgroups act to secure and enhance a balanced immune response. In other words, the optimal scenario would seem to be that humans should produce a well-balanced Th1/Th2 response suited to fight the immune challenge. (Gupta and Gupta 2020; Kool et al. 2008).

For effective vaccination, selecting the appropriate adjuvant type for robust antibody generation and the desired antigen-specific solid immune response is essential for vaccine development. The incorporation of a conventional adjuvant along with antigens in vaccine formulation is a well-established practice in experimental immunology. Aluminum hydroxide-based suspensions are the most used adjuvants.
For example, the 2% Aluminum hydroxide (Alum) wet gel suspension Alhydrogel adjuvant has been approved as an adjuvant in multiple licensed vaccines for human use in many countries worldwide (Morefield GL. et al. 2005).

The mechanism of Alhydrogel action is complex. However, depot formation at the injection site has been shown, allowing for a slow antigen release. This technology prolongs the interaction time between the antigen and the antigen-presenting cells (APCs) (Sutterwala FS, Flavell R.A. 2008). In addition, Alhydrogel is generally known to provoke a robust Th2 response, directly stimulating monocytes to produce pro-inflammatory cytokines (Kool et al. 2008). However, the use of Alum as an adjuvant has several limitations. First, the Aluminum adjuvant-containing vaccines can cause post-immunization headaches, arthralgia, and myalgia (Lindblad 2004; Lew et al. 1988).

The number of modern adjuvants approved for clinical use in humans is limited. For example, no peptide adjuvants have been registered for human use.

Some main requirements for a new generation of adjuvant activities are described in (Gupta and Gupta 2020). Therefore, a novel adjuvant needs to be developed to produce vaccines that cannot currently be adjuvanted by Alum-based vaccination strategies (Geerligs et al. 1989).

A new promising direction in developing a new generation of adjuvant is the use of immune- and hemopoietic peptides. A more intensive study of peptide preparations involved in regulating the immune response and hematopoiesis will pave a new prospect for its use as effective and safe adjuvants.

Isolation of the biologically active peptide Glu-Trp from the thymus gland (Morozov V.G. Khavinson, V.K. 1997) was followed by a series of structure-functional studies (SAR) of related linear and cyclic peptides (Deigin, V.I. et al. 1999, Deigin, V.I. et al. 2007, Deigin, V.I. et al., 2000, Semina, O.V. et al. 1997, Vinogradova, Yu et al. 1999).

As a result, an original tripeptide Ile-Glu-Trp, which showed hemostimulating activity, stimulating the proliferation of hemopoietic stem cells damaged by radiation or cytokstatic action, has been discovered.

A study of the pharmacokinetics of H3-(Ile-Glu-Trp) showed that the intramuscularly administered peptide predominantly accumulates in bone marrow (45%) (Semina, O.V. et al. 1997). Therefore, a medicinal product based on this peptide is registered in Russia as an immune-and hemostimulatory agent under the trade name Stemokin® (Deigin, V.I. et al. 2007).

Unlike the known peptide immunomodulators Zadaxsen (E.Garachi. 2007), Immunox (Goldstein et al. 1979), Thymogen (Deigin, V.I. et al. 1999), Likopid (Meshcheryakova et al. 2007), Stemokin acts on earlier stages of hematopoiesis, including CD34 cells, at the same level as other hemoregulatory Granulocyte- and Granulocyte-macrophage colony-stimulating factors (CSF, G-CSF, and GM-CSF) (Semenets, T.N. et al. 2000).

The experimental and clinical information available for Stemokin (Deigin, V.I. et al. 1999, Vladislav Deigin, et.al., 2020, Deigin, V.I. et al. 2007, Deigin V. 2012, Deigin V.I et al 2016, Deigin, V.I. et al., 2000, Semina, O.V. et al. 1997, Vinogradova, Yu et al. 1999) is applied to undertake the first pilot proof of concept experiment directed to use Stemogen as a “modern adjuvant” (Gupta and Gupta 2020).

This study aims to compare the potential adjuvant activity of Stemokin versus the approved adjuvant Alhydrogel in a murine vaccination model with the approved VAXIGRIP® Vaccine (Hannoun 2013). Vaxigrip vaccines (Vaxigrip®, IIV3, and VaxigripTetra™ IIV4) from SanofiPasteur are registered flu-preventing vaccines (Hannoun 2013); this Vaccine contains Alhydrogel as an adjuvant. In addition, in the 1980s, the trivalent Vaccine Vaxigrip™ – Inactivated Influenza Vaccine, Trivalent Types A and B, was developed (Viviane Gresset-Bourgeois et al. 2017). Vaxigrip influenza vaccines provide an opportunity to reduce further the morbidity and mortality associated with annual influenza epidemics.

Materials & Methods

Animals

The procedures performed in this study followed the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health with the “Regulations for Studies with Experimental Animals” (Decree of the Russian Ministry of Health from Aug 12, 1997, No. 755). The Institutional Ethics approved the protocol. Committee of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry (Protocol No.161/2015). All efforts were taken to minimize suffering. Balb/c female mice are aged 9–11 weeks (Harlan Laboratories, Canada). The initial weight range for use in the study was 19–21 g. The mice were housed in microisolator cages. Mice were s.c. injected with 100 µl/mouse. All groups (10 mice in a group) received one administration on Day 1 and Day 28.

Test Article

Stemokin Sodium Chemical Name: L-Isoleucyl-L-Glutamyl-L-Tryptophan Monosodium salt.

Purity: 99.8% (LCMS UPLC Waters Acuity System). (Immunotech Developments Inc. Canada).
Influenza Vaccine

Vaxigrip is an inactivated influenza vaccine trivalent Types A and B (split virion), manufactured and distributed by Sanofi Pasteur Limited, Toronto, Canada. It is prepared from a virus grown in the allantoic cavity of embryonated eggs. The virus is purified by zonal centrifugation on a sucrose gradient, dissolved in the surfactant octoxinol 9 (Triton® X100), inactivated in formaldehyde, and then diluted in phosphate-buffered saline. For the experiment, each 0.5 mL dose of Vaxigrip® contains 15 µg HA A/B (H1N1)-like strain and 15 µg HA A/B (H3N2)-like strain. Other Ingredients are ≤ 30 µg formaldehyde, up to 0.5 mL sodium phosphate-buffered, isotonic sodium chloride solution, 2 µg thimerosal as a preservative, and the surfactant Triton® X100. There is no adjuvant presented.

Adjuvant: Aluminum Hydroxide

Name: “Alhydrogel”; (Accurate Scientific, USA).

Scheme of the Experiment

Formulation of the Test Articles

Stock Solutions (20 mg/mL and 0.5 mg/mL) of Stemokin were prepared in sterile, non-pyrogenic saline. The solutions were filtered through a sterile, non-pyrogenic PVDF membrane filter with porosity NMT 0.2 µm. 222 µL of Vaxigrip suspension was diluted with the saline to make 2000 µL solution (group 1) or diluted with to make 2000 µL of solution after adding the calculated amount of stock solutions of Stemokin or Alhydrogel (groups 2–5).

Mice Treatment

Ten mice each were randomized into treatment groups. A 100 µL of the appropriate Formulation for the allocated group was injected as a bolus subcutaneously on Day 1 and then again on Day 28.

Blood Collection

Blood was collected on days 14, 28, and 42 from the saphenous vein without anticoagulants, and serum was separated and stored at –70°C until use in serological assays.

IgG Isotype ELISAs (IgG)

Briefly, ELISA plates were directly coated with the Vaxigrip. Next, dishes were washed with PBS containing 0.05% Tween 20 (T-PBS) and blocked with 1% bovine serum albumin (BSA) for one h at 37 °C. Next, antigen-coated plates were washed with T-PBS and incubated with 1:1,000-diluted individual mice serum samples overnight at 4 °C. After washing with T-PBS, plates were incubated with goat anti-mice IgG1 or IgG2a horseradish peroxidase conjugates (Rockland Immunochemical) in a 1:10,000 dilution for 2 h at 37 °C. The reaction was developed by o-phenylenediamine for 30 min and read the optical density at 450 nm. Influenza-specific ELISA titers were extrapolated by linear regression from a standard curve and expressed as ug/ml. Averages were presented for both IgG isotypes.

Hemagglutinin Inhibition (HI) test

Mouse serum samples were treated with receptor destroying enzyme (RDE) at 37 °C overnight. Fresh turkey red blood cells (TRBC) were washed and diluted in PBS to a concentration of 0.5% (vol/vol). The mice sera were diluted in PBS in 96-well V-bottom cell culture plates. The serially diluted pool sera from each mice group were incubated with the H3N2 Influenza strain for 15 min. Fifty microliters of 0.5% TRBC were then added, and the plates were incubated at room temperature for 30 min. The hemagglutination inhibition (HI) titer was the reciprocal of the highest serum dilution to prevent agglutination completely. The log₂ transformed geometric mean titers for each group are presented.

Statistical Analysis

The student’s t-test has been used to compare data differences in antibody concentrations and titers for each mice group vs. V and V+Alum groups. The data presented as x ± SEM.
At the same time, it was no difference in the concentration of IgG1 on day 42 for all groups. The different response was with IgG2a amounts; on day 42, the concentration of the IgG2a in all groups received (V + S) was higher than for the (V + Alum) group. Thus, the mice group received (V + Alum) demonstrated an early IgG1 response, while the induction of IgG2a antibodies can only be stimulated by animals that received V with Stemokin at various doses. The Alhydrogel treated group showed early stimulation of a Th2 response, consistent with promoting humoral immunity in preference to a cellular response. Successively high Stemokin dose in the vaccine composition produced progressively higher Vaxigrip + Alhydrogel IgG2a response.

This pilot experiment shows that Stemokin has the potency of a vaccine adjuvant favoring a balanced Th1/Th2 response represented as IgG1 and IgG2a in the combined Vaccine/Stemokin group compared to the Vaccine + Alhydrogel group (Fig. 2).

To evaluate the Hemagglutinin Inhibition (HI) ability by the Stemokin (V + S100) and Alhydrogel (V + Alum) groups, we tested mouse serum samples against the H3N2 Influenza strain. The HI titer was the reciprocal of the highest serum dilution to prevent agglutination completely. The log₂ transformed geometric mean titers for each group are presented in Fig. 3. All studied groups on day 42 showed high HI activity, and there was no significant difference between the sera of all studied groups; however, the high dose of the Stemokin adjuvant group (V + S 100 µg) on Day 42 tends to inhibit the HI titers compared to Vaccine + Alhydrogel (V + Alum) group.

Mice were immunized in three variants with Vaxigrip - without adjuvants, Alhydrogel adjuvant, and three doses (1, 25, and 100µ) of Stemokin as an adjuvant. (Fig. 1).

This experiment estimates anti-influenza antibodies of IgG1 and IgG2a isotypes and Stemokin adjuvanticity compared to Alhydrogel in inducing specific anti-hemagglutinin antibodies after Vaccine immunization.

At a different time, the data shows that Stemokin treated groups boosted the IgG2a antibody response two weeks earlier than the Aluminum (V + Alum) group.

As shown in Fig. 1, on day 28 after the first immunization, the (V + Alum) mice group stimulated significantly higher IgG1 levels than the V and (V + S100 µg) groups.

**Results**

Pilot experiments to evaluate the adjuvant potential of Stemokin have been performed in an experimental murine model with Influenza Virus Vaccine (Vaxigrip™) as an immunogen (Hannoun 2013). The approved Alhydrogel adjuvant has been used as a comparison preparation (Kool et al. 2008).

At the same time, it was no difference in the concentration of IgG1 on day 42 for all groups. The different response was with IgG2a amounts; on day 42, the concentration of the IgG2a in all groups received (V + S) was higher than for the (V + Alum) group. Thus, the mice group received (V + Alum) demonstrated an early IgG1 response, while the induction of IgG2a antibodies can only be stimulated by animals that received V with Stemokin at various doses. The Alhydrogel treated group showed early stimulation of a Th2 response, consistent with promoting humoral immunity in preference to a cellular response. Successively high Stemokin dose in the vaccine composition produced progressively higher Vaxigrip + Alhydrogel IgG2a response.

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et al. 1975). Peptidoglycan MDP and most analogs enhance humoral antibody production, demonstrated with classical laboratory antigens and conventional vaccines against viral, bacterial, or parasitic pathogens (Siddiqui, W.A. et al. 1978). In addition, it is reported that MDP (Girardin et al. 2003) and their disaccharide derivative GMDP (Meshcheryakova et al. 2007) confer their adjuvanticity by activating the NF-κB pathway through the NOD2 receptor.

Another application of peptide adjuvants is peptide amphiphile micelles (PAMs). These compounds have been studied as vaccine carriers, inducing strong antibody response (Barrett J.C. et al. 2017, Trent et al. 2015). The literature (Zhang R 2018) shows that a single immune agonist is not always sufficient to elicit an efficacious immune response. Usually, Aluminum hydroxide-based adjuvant vaccines are directed to prime the Th2 immune response rather than a Th1-biased response. Composite adjuvants containing Aluminum and immunomodulatory peptides could be used to enhance the immune response (Chang T.Z. et al. 2017, Deng et al. 2018) synergistically. According to the specific immune response generated by different adjuvants, several composite adjuvant systems might find clinical applications (Wang et al. 2017, Sutherland D.R. et al. 1996).

Discussion

The critical factor in vaccine design is ensuring the Vaccine’s efficacy while reducing the potential risks associated with it.

The immune response induced by antigenic or genetic immunization can thus generally be distinguished by these two subtypes: Th1/Th2. Successful vaccines for most pathogens will require enhanced immune responses, including Th1-cellular-mediated immunity and robust Th2-humoral response. It is necessary to vaccinate twice or three times to obtain an intense and long-lasting immune response. An essential mechanism in the immune regulation involves homeostasis between the Th1 and Th2 activity of CD4+ T helper cells expressing different cytokine patterns. (Gupta and Gupta 2020; Kool et al. 2008).

Adjuvants may exhibit their immunostimulatory effects via various mechanisms: providing antigen depot; activation of innate immunity through pathogen recognition receptors engagement; co-stimulation of immune cells; and immunomodulation, e.g., maturation of particularly dendritic cells (Joffre O.P. et al. 2012).

Various experimental studies are performed with peptides as potential adjuvants.

In 1975, the active subunit of bacterial cell walls in Freund’s complete adjuvant was identified and synthesized (Audibert et al. 1982; Ellouz et al. 1974, Kotani, S. et al. 1975). Peptidoglycan MDP and most analogs enhance humoral antibody production, demonstrated with classical laboratory antigens and conventional vaccines against viral, bacterial, or parasitic pathogens (Siddiqui, W.A. et al. 1978). In addition, it is reported that MDP (Girardin et al. 2003) and their disaccharide derivative GMDP (Meshcheryakova et al. 2007) confer their adjuvanticity by activating the NF-κB pathway through the NOD2 receptor.

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Composite adjuvants containing Aluminum and immunomodulatory peptides could be used to enhance the immune response (Chang T.Z. et al. 2017, Deng et al. 2018) synergistically. According to the specific immune response generated by different adjuvants, several composite adjuvant systems might find clinical applications (Wang et al. 2017, Sutherland D.R. et al. 1996).
Most composite adjuvants are heterogeneous emulsions, leading to local and systemic adverse reactions (Lindblad 2004; Lew et al. 1988).

In addition to a more balanced immune response, Stemokin solution as an adjuvant does not cause local complications compared to most composite adjuvants. Because Stemokin acts on earlier stages of hematopoiesis, including CD34 cells, stimulating the proliferation of hematopoietic cells at the same level as other hemoregulatory factors we have compared the Stemokin effect with (G-CSF and GM-CSF) (Francisco-Cruz et al. 2014; Souza et al. 1986) on hematopoietic cells. After treatment by Stemokin the total bone marrow cells amount was restored faster than the GM-CSF group. Furthermore, the cellularity volume in mice treated with Stemokin completely recovered two times more intensive than in the group treated with GM-CSF (Semenets, T.N. et al. 2000).

The close interaction of the hematopoiesis and immune system (Na Li et al. 2017), we have evaluated the Stemokin adjuvanticity compared to Alhydrogel.

The segregation of IgG1 and IgG2a immunoglobulin isotypes as markers for Th2 and Th1 lymphocytes, respectively, was investigated.

For this study, the influenza vaccine Vaxigrip was used to immunize mice in three variants - without adjuvants, Alhydrogel adjuvant, and three Stemokin doses.

After the first immunization, the (V + Alum) treated group on Day 28 elicited significantly higher IgG1 levels than the V and (V + S100 µg) groups. However, at the further immunization, the difference in the concentration of IgG1 on day 42 for all groups was insignificant.

At the same time, the concentration of the IgG2a in all groups received (V + S) on day 42 was higher than for the (V + Alum) group. The mice group received (V + Alum) demonstrated an early IgG1 response, while the induction of IgG2a antibodies can only be stimulated by animals that received (V + S1 µg), (V + S 25 µg), and (V + S100 µg) doses.

These experimental results showed the early stimulation of Th2 response by the Alhydrogel treated group consistent with promoting humoral immunity in preference to a cellular one. At the same time, successively high Stemokin dose in the vaccine composition produced progressively higher IgG2a response comparable to Vaxigrip + Alhydrogel IgG1 response.

Using a Stemokin in the composition makes it possible to generate a substantial humoral and T-cell response, balancing Th1/Th2 better than generally obtained by standard Alhydrogel (V + Alum) vaccine adjuvant use.

**Conclusion**

This first proof-of-concept pilot experimental study provides evidence that a pharmaceutical preparation Stemokin has the potency of a vaccine adjuvant favoring a balanced Th1/Th2 response.

Following Stemokin treatment, anti-HA IgG2a response was enhanced, revealing a Th1-favoring balanced Th1/Th2 immunomodulation. In other words, Stemokin promotes a more rapid protective response.

The optimized Stemokin concentration in the specific immunogen/adjuvant composition can generate a higher IgG2a (Fig. 1) response and balance the Th1/Th2 ratio more efficiently than a traditional vaccine adjuvant.

More detailed studies of Stemokin and its analogs as adjuvants are planned with various types of modern vaccines.

**Authors Contributions** Vladislav Deigin: Peptide synthesis, wrote the main manuscript text. Dmitry Koroev: prepared all figures and statistical analysis. Olga Volpnia: immunological testing, wrote the main manuscript text.

**Declarations**

**Competing Interests** The authors declare no competing interests.

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