Effect of a combination of glutamyl-tryptophan and glycyrrhizic acid on the course of acute infection caused by influenza (H3N2) virus in mice

V. S. Smirnov¹, V. V. Zarubaev², P. M. Anfimov², and A. A. Shtro²

¹ ZAO “CytoNIR”
² Research Institute of Influenza, Ministry of Health and Social Development of Russia, Saint Petersburg

The purpose of the study was to evaluate the modulating effect of glutamyl-tryptophan (EW), glycyrrhizic acid (GA), and their combination on the course of experimental infection caused by influenza A (H3N2) virus in mice. The animals were infected with influenza A/Aichi/2/68 (H3N2) virus in a dose of 1 or 10 LD50. GA (10 mg/kg body weight) and EW (0.1, 10, and 1000 µg/kg) alone or in combination were intraperitoneally injected for 5 days, starting on day 1 of virus infection. Rimantadine 50 mg/day was used as a comparison drug. The combination of EW (1000 µg/kg) was ascertained to exert the maximum protective effect manifesting itself in reducing the death of infected animals (by 75-79% compared to the control depending on the viral dose) and the titers of viruses accumulated in the lung (5-6 log EID50) and in preventing lung tissue edema and inflammation. The noted effect was comparable with that seen in the use of rimantadine. The agents used alone had a lower efficacy than rimantadine. The finding permit the combination of GA and EW to be considered to be a promising agent for the treatment of influenza.

Keywords: glutamyl-tryptophan, glycyrrhizic acid, combined drug, influenza, antivirals

Contact information: Smirnov, Vyacheslav Sergeevich, Dr. Sci. (Med.), Prof., Chief Researcher; e-mail: vssmi@mail.ru
Every year influenza viruses cause disease outbreaks all around the globe. Although school children are most susceptible to the disease, influenza viruses also lead to higher mortality rates among older population during the flu season [7]. There is a great amount of evidence testifying to the relationship between influenza outbreaks and the number of hospitalizations and deaths caused by acute pneumonia, chronic cardiopulmonary diseases and other conditions that might evolve as an influenza complication [10, 11].

The best practice of raising resistance to the infection is anti-flu vaccination; however, a vaccine is only effective against a flu strain with a specific group of antigens [9]. Despite the advanced system of influenza monitoring, a new type of virus may evolve and spread very fast and may not match the type of strain included in the vaccine.

Anti-influenza drug therapy also plays an important role in influenza treatment. Although etiotropic pharmaceuticals, such as rimantadine, ozeltamivir and ribavirin, can reduce disease severity and duration, these drugs are toxic and have a number of side effects [2, 14]. In addition, influenza viruses can become resistant to some of the most popular antivirals, for example, derivatives of adamantane (amantadine and rimantadine) and ozeltamivir [12]. Thus, new antiviral strategies are required to control influenza viruses.

Immunomodulating agents represent one of such novel approaches [4, 8]. Among some of the compounds used for influenza treatment and prevention is dipeptide glutamyl-tryptophan (EW) (more commonly known as thymogenum [6]), bendazol and combination drug Cytovir-3 [4, 5], multiple interferon-inducing agents [1], monoclonal antibodies of various specificity [8], as well as glycyrrhiza extracts and their derivatives [15]. The available data [4] suggest that the majority of the above drugs are most effective as urgent prevention measures used either within the latent stage or during the first hours of the disease. As the infection progresses, the efficacy of the drugs decreases, although even at this stage antivirals can significantly reduce the possibility of post-infection complications. Efficacy studies of single agent thymogenum and bendazol versus their combination showed that the combination drug was superior to each of the components used as a single agent [4]. These results correlate with the common opinion that a combination of two compounds that modulate different immune reactions is more effective than each of the components used separately [8]. Therefore, of great interest is the combination of thymomimetic EW (supposedly the agonist of metabotropic glutamate receptors) and glycyrrhizic acid that inhibits the expression of inflammatory cytokines by inhibiting the activity of Toll-like receptors TLR-3 and TLR-4 [13]. The ability of these agents to decrease the severity of influenza in experimental groups has been well documented [4, 15]. In view of the foregoing, great interest lies in the in vivo study of the anti-influenza activity of glycyrrhizic acid trisodium salt (GATS), EW and their combination administered as a treatment regimen in animals with lethal influenza pneumonia at the stage of the clinical manifestation of the disease.

Materials and Methods

Experimental agents. GATS and glutamyl-tryptophan (EW) sodium salt (by Medical and Biological Research and Production Enterprise “Cytomed” CJSC, St. Petersburg) were used in the study. Rimantadine (α-methyl-1-adamantyl-methylamine hydrochloride, by Aldrich Chem. Co., Milw., WI, cat. #39.059-3) was used as a reference.

Virus. Mouse adapted A/Aichi/2/68 (H3N2) virus was passaged for 48 hours in the allantoic cavity of embryonated hens’ eggs (10-12 days old) at 36°C.

Animals. White female outbred mice weighing 12-16 g were provided by “Rappolovo” breeding nursery (Leningrad Region) and were on a standard diet in the vivarium of the Research Institute of Influenza under the Russian Academy of Medical Sciences. The animals for the experimental groups were selected randomly and were observed for one week before study initiation.

Experimental influenza infection. The animals were inoculated with the allantoic fluid of embryonated hens’ eggs containing the virus. A series of 10-fold dilutions was performed with saline solution, followed by a separate experiment to determine the infectivity of the material by virus titering against animal mortality rates. Reed and Mench method was used to calculate the virus titer.

The experimental agents (0.2 ml) were administered intraperitoneally once a day for 5 days beginning from the first day after inoculation. The same treatment regimen was used for the reference drug. Drug dosing: 10 mg of GATS per 1 kg of animal weight; EW 1000, 10 and 0.1 mg/kg; rimantadine 50 mg/kg. Phosphate buffered saline was used as a placebo in the control group. A group of intact animals were used as negative control and were kept in the same conditions as the experimental animals.

The virus was administered to the lightly etherized mice intranasally in the 1 LD₅₀ (25 mice per group) and 10 LD₅₀ (20 mice per group). On the third day after inoculation 10 animals out of each group were sacrificed and autopsy was performed to isolate the...
lungs. Five out of 10 lungs were used to isolate the virus (samples were frozen and stored at -20°C until time of testing), while the remaining 5 lungs were fixed in 10% buffered formalin to be later used for histology (see below). The lungs of animals infected with 10 LD₅₀ were used only for virus isolation.

Other animals were observed for 14 days during which daily weight measurements and mortality assessments were performed in the control and experimental groups.

The obtained animal mortality data in each group were used to calculate the mortality index (M), the protective index (IP) and the median day of death (MDD) taking into account the 14-day observation period. The following formulae were used:

MDD = (Σ N × D)/Nt,
where N is the number of animals surviving for D days, and Nt is the total number of animals in a group;

M = M/Nt,
where M is the number of animals (in a group) dying during the 14-day period after inoculation;

IP = ((Mc-Me)/Mc) × 100%,
where Mc and Me indicate the mortality (%) in the control and experimental groups, respectively.

Lung tissue virus titering. In order to determine the infectious titer of the influenza virus in the mice lung tissue, the lungs isolated on the third day after inoculation were homogenized in the 10-fold volume of sterile phosphate buffered saline, and the obtained homogenates were used for a series of 10-fold dilutions with the same buffer solution. The influenza virus titer was calculated using the MDCK cell culture (ATCC # CCL-34) grown on the 96-well plates in MEM. The cells were infected with 10-fold dilutions of the lung homogenate (10⁻⁸-10⁻⁵) and were treated in an incubator for 48 hours. Following incubation the culture fluid was transferred onto the plate wells for immunologic reactions and an equal volume of 1% suspension of hen red blood cells (RBC) in a saline solution was added.

The rate of virus replication in the plate wells was assessed by the level of RBC hemagglutination. In the study the virus titer was calculated as the opposite of common logarithm of the value of the biggest virus dilution giving a positive result in the hemagglutination test, and was expressed as logarithm 50% of experimental infectious virus dose (lg EID₅₀).

Histology. To perform structural analysis the lungs were fixed in 10% phosphate-buffered formalin and the paraffin-embedded histology samples were prepared according to the standard procedure. Dewaxed sections were stained with hematoxylin and eosin. The resulting samples were analyzed for intensity and cell content of the inflammatory infiltrate found in the sites damaged due to pneumonia; the severity of degenerative and proliferative processes in the lung tissue was also evaluated.

Broncholith and pulmonary edema were assessed semiquantitatively using the stained sections of the lung tissue according to the following criteria: 0 – cells take up 0-25% of the field of view (typical of intact lungs); 1 – 25-50% (moderate edema), 2 – 50-75% (severe edema), 3 – 75-100% (total pulmonary edema). In each group the samples were analyzed in 10-20 fields of view.

Statistical data analysis. Statistical analysis of data (calculation of median values and standard deviations) was performed in Microsoft Excel. The statistical significance was assessed according to the Student’s criterion. The differences between groups were considered as significant if: p ≤ 0.05.

Results

Protective efficacy of investigational agents in animal studies. Non-specific mortality of animals was not reported in the intact control group. The clinical signs of the disease were typical for the influenza infection, including difficulty breathing, ataxia, tremor, and decreased food and water intake.

A summary of mortality dynamics in the animal control and experimental groups, levels of virus replication in lung tissue, as well as the influence of the agents on the severity of inflammation and lung edema are presented in the table.

Inoculation of animals with the virus lead to dose-dependent mortality reported in the experimental groups beginning from Day 6 after inoculation. Glycerzrhizic acid trisodium salt, glutamyl-tryptophan and rimantadine reduced mortality rate and prolonged animal survival. The highest efficacy was evident with the maximum dose of EW (1 mg/kg) and the combination of EW and GATS (protective indices 41 and 71%, respectively), the latter exceeding the efficacy of rimantadine.

Effect of experimental agents on virus replication. Depending on the infectious dose, the model virus yielded titers of 6.9-7.3 lg EID₅₀/20 mg of lung tissue. GATS did not affect virus replication in the tissue, but the use of EW led to statistically significant inhibition of virus activity in the lungs, both with or without GATS. This was true in case of both low and high virus infectious doses.

Pathological analysis. Beginning from Day 6 after the inoculation, the following structural changes were found in the lung tissue of non-treated infected mice:
neutrophil and cell debris accumulation in the lumen of big bronchi, virus-specific damage of bronchial epithelial cells with the formation of virus inclusions and the presence of cast-off cells in the bronchial lumen, acute edema due to interstitial accumulation of serous fluid, areas of hemorrhagic edema, neutrophil infiltration, cell lysis in the respiratory portions of lungs, dilation of blood vessels and atelectasis (Figure 1, see page 2 of the cover). A substantial part of neutrophils was undergoing the destruction stage. The abovementioned phenomena are typical for acute viral pneumonia, and their intensity may be used to determine the disease severity grade.

In the animals receiving the investigational agents the structure of the lung tissue was similar to that of the control group. The main difference from the non-treated animals lay in the dramatic regression of signs of virus-specific and reactive damage of the lung tissue during acute influenzal pneumonia. Thus, on Day 6 after the animals had been infected the bronchial epithelial cells seemed intact (Figure 2a, see page 2 of the cover), unlike the lysed cells with multiple virus inclusions in the tissue of the control animals. The area of inflammation was smaller than that reported in the control group (Figure 2b). The results showed that the best recovery of the lung tissue structure could be expected from the combination of EW (1000 µg/kg) and GATS (Figure 3a, see page 2 of the cover), and its activity was comparable with that of rimantadine (Figure 3b). Other drug combinations had similar or less effect on the lung structure during influenzal pneumonia.

Microscopic examination of lung tissue was also used for the quantitative assessment of lung impairment in the control and experimental groups. According to the results presented in the table, the best efficacy in treating edema and inflammatory infiltration in the lungs was reported with the combination of GATS and EW (1000 µg/kg), which correlates with the findings of the tests described above.

**EW and GATS effect on influenza pneumonia caused by influenza A/Aichi/2/68 (H3N2) virus in white mice**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Virus dose, LD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MDD, days</th>
<th>Mortality, %</th>
<th>Protective indices, %</th>
<th>MDD increase, days</th>
<th>Virus titer on Day 3, lg EID&lt;sub&gt;50&lt;/sub&gt;/mg of tissue</th>
<th>Severity of lung edema, grades</th>
</tr>
</thead>
<tbody>
<tr>
<td>EW, 0.1 µg/kg</td>
<td>10</td>
<td>8.4</td>
<td>93.3</td>
<td>0.0</td>
<td>-0.3</td>
<td>6.5 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11.9</td>
<td>46.7</td>
<td>12.5</td>
<td>0.5</td>
<td>5.2 ± 1.2</td>
<td>1.7 ± 0.2 (p=0.002)</td>
</tr>
<tr>
<td>EW, 10 µg/kg</td>
<td>10</td>
<td>9.8</td>
<td>80.0</td>
<td>14.3</td>
<td>1.1</td>
<td><strong>2.7 ± 1.0</strong></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12.3</td>
<td>46.7</td>
<td>12.5</td>
<td>0.9</td>
<td>0.9 ± 0.3</td>
<td>3.2 ± 0.2 (p=0.092)</td>
</tr>
<tr>
<td>EW, 1000 µg/kg</td>
<td>10</td>
<td>11.5</td>
<td>60.0</td>
<td>35.7</td>
<td>2.9</td>
<td>2.3 ± 0.9</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>13.4</td>
<td>26.7</td>
<td>50.0</td>
<td>2.1</td>
<td><strong>1.8 ± 1.0</strong></td>
<td>1.1 ± 0.2 (p=0.000)</td>
</tr>
<tr>
<td>GATS, 10 mg/kg</td>
<td>10</td>
<td>9.9</td>
<td>80.0</td>
<td>14.3</td>
<td>1.3</td>
<td>7.0 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12.0</td>
<td>53.3</td>
<td>0.0</td>
<td>0.7</td>
<td>5.8 ± 1.0</td>
<td>2.1 ± 0.2 (p=0.073)</td>
</tr>
<tr>
<td>GATS + EW, 0.1 µg/kg</td>
<td>10</td>
<td>9.9</td>
<td>86.7</td>
<td>7.1</td>
<td>1.2</td>
<td>6.9 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12.2</td>
<td>53.3</td>
<td>0.0</td>
<td>0.9</td>
<td>5.6 ± 1.3</td>
<td>1.0 ± 0.2 (p=0.000)</td>
</tr>
<tr>
<td>GATS + EW, 10 µg/kg</td>
<td>10</td>
<td>12.1</td>
<td>53.3</td>
<td>42.9</td>
<td>3.4</td>
<td><strong>2.0 ± 1.3</strong></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12.5</td>
<td>33.3</td>
<td>37.5</td>
<td>1.2</td>
<td><strong>1.5 ± 1.3</strong></td>
<td>2.2 ± 0.2 (p=0.112)</td>
</tr>
<tr>
<td>GATS + EW, 1000 µg/kg</td>
<td>10</td>
<td>13.9</td>
<td>20.0</td>
<td>78.6</td>
<td>5.2</td>
<td><strong>1.4 ± 0.5</strong></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>14.1</td>
<td>13.3</td>
<td>75.0</td>
<td>2.7</td>
<td><strong>1.6 ± 0.9</strong></td>
<td>0.9 ± 0.1 (p=0.002)</td>
</tr>
<tr>
<td>Rimantadine, 50 mg/kg</td>
<td>10</td>
<td>13.0</td>
<td>26.7</td>
<td>71.4</td>
<td>4.3</td>
<td><strong>2.2 ± 0.8</strong></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>13.9</td>
<td>13.3</td>
<td>75.0</td>
<td>2.6</td>
<td><strong>2.3 ± 0.9</strong></td>
<td>0.9 ± 0.2 (p=0.000)</td>
</tr>
<tr>
<td>Virus control</td>
<td>10</td>
<td>8.7</td>
<td>93.3</td>
<td>-</td>
<td>0.0</td>
<td>7.3 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11.3</td>
<td>53.3</td>
<td>-</td>
<td>0.0</td>
<td>6.9 ± 0.4</td>
<td>2.7 ± 0.2 (p=1.000)</td>
</tr>
</tbody>
</table>
Discussion

The results of the study have proved the justifiability of the initial hypothesis regarding the higher efficacy of a combination drug (consisting of EW and GATS) as compared to the two components used as single agents. The combination of 10 mg/kg GATS and 1 mg/kg EW yielded slightly higher survival of animals as compared to the rimantadine group. The higher survival rate, associated with the treatment by the investigational combination drug, was followed by a marked tendency of lower virus titer in the lungs and the respective recovery of the normal lung structure as seen during the histology.

Dipeptide derivatives are being actively developed as antiviral agents. The greatest interest lies in the dipeptides that have target cells, exert a mediated impact on the virus infection, and, thus, involve immunomodulation, induction of interferon, etc. [3]. Such compounds do not demonstrate apparent antiviral activity in the cell cultures, but are highly effective in animal studies, which can be explained by the mechanism of action that involves the interaction of multiple cell cultures. In this context EW, which was used in the above study, belongs to a group of agents that interact with the innate immunity. Therefore, the combination of EW and GATS is fully justifiable, because glycyrrhiza derivatives also inactivate the pattern recognition receptors, though different from those used by EW. To conclude, the combination of the two compounds helps to exert the maximum effect on the innate immunity which is inactivated to fight against a pathogenic virus. The results suggest that the combination of GATS and EW has the potential of becoming an anti-influenza drug.

REFERENCES

Received on 30.11.11
Fig. 1. Severe hemorrhagic edema (a) and degradation of bronchial epithelium (b) developing as a result of influenza pneumonia caused by the A/Aichi/2/68 (H3N2) virus in a white mouse; condition on Day 6 after inoculation.

Inflammation-related effusion and cell debris accumulation in the lumen of bronchi. Samples stained with hematoxylin and eosin, × 200.

Fig. 2. Lung tissue structure of a white mouse with influenza pneumonia (caused by the A/Aichi/2/68 (H3N2) virus) on Day 6 after inoculation; mouse treated with EW 1000 µg/kg (a) and 0.1 µg/kg (b).

Bronchial epithelium is intact, moderate edema of interalveolar septum. Samples stained with hematoxylin and eosin, × 200.

Fig. 3. Lung tissue structure of a white mouse with influenza pneumonia (caused by the A/Aichi/2/68 (H3N2) virus) on Day 6 after inoculation; mouse treated with EW (1000 µg/kg) combined with GATS (10 mg/kg) (a), and rimantadine (50 mg/kg) (b).

Bronchial epithelium is intact, mild edema of interalveolar septum and mild cellular infiltration. Samples stained with hematoxylin and eosin, × 200.