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Utilization of alpha-1-acid glycoprotein levels in the serum as a parameter for in vivo assay of influenza virus inhibitors. Antivir Chem Chemother

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Alpha-1-acid glycoprotein (AGP), an acute phase protein in serum assayed by single radial immunodiffusion using a commercially available kit, was found to significantly increase in mice infected with influenza A and B viruses. Experiments were run to determine the rate of increase of serum AGP and its relation to other influenza disease parameters, including lung consolidation, development of lung virus titres, decline in arterial oxygen saturation ($\text{SaO}_2$), histopathological changes in the lung, and death of the animal. Maximal AGP levels occurred by day 3 in the animals, at about the same time lung virus titres reached their peak and inflammatory effects were evident in the lung. Serum levels of AGP were then compared with other disease parameters in the evaluation of the anti-influenza A and B virus efficacy of oseltamivir and ribavirin in mice. Treatment was by oral gavage twice daily for 5 days, beginning 4 h before virus exposure using doses of 100, 10, and 1 mg/kg per day of oseltamivir and 75 mg/kg per day of ribavirin. Against the influenza A infection, significant inhibition of death, $\text{SaO}_2$ decline, and lung consolidation was seen at all doses of each compound; day-6 AGP levels were reduced in a dose-responsive manner. Lung virus titres were lessened at this time, but to a significant degree only at the high dose of oseltamivir and by ribavirin. The influenza B virus infection, which appeared more severe than the influenza A infection, was also significantly inhibited by both compounds, but to a lesser extent. The serum AGP levels were again lessened by therapy with both compounds. The influence of challenge dose of influenza A virus on AGP level and on the antiviral activity of 20 mg/kg per day of oseltamivir, administered by oral gavage, was determined in mice. The AGP level was in proportion to the viral challenge dose; oseltamivir significantly inhibited AGP levels and all other disease parameters regardless of size of viral inoculum. These data indicate murine AGP levels to be markedly stimulated by infection with influenza A and B viruses, and the level of the protein to be an additional measure of antiviral efficacy.

**Keywords:** alpha-1-acid glycoprotein, influenza virus, antiviral, oseltamivir
fluid in the lungs, but does not reflect the condition of the lung tissue. Histopathological analysis is cumbersome and difficult to quantitate. Only arterial oxygen saturation (SaO2) as measured by pulse oximeter, has provided a quantitative and reproducible assessment of lung condition, although it can only be used in albino mice, since poor light transmission through the vascular bed occurs in pigmented animals (Sidwell et al., 1992). Also, SaO2 decline is less apparent in animals infected with low viral challenge doses. Thus, measurement of serum AGP levels may provide an additional quantitative assessment of alleviation of tissue injury in infected, drug-treated animals.

A kit for the quantitation of this protein using radial immunodiffusion has recently become commercially available (Saikin Kagaku Institute, Sendai, Japan), providing a means of readily determining serum levels on a standardized basis. Since the studies by Wong et al. (1989) utilized rocket immunoelectrophoresis using AGP antisera prepared in their laboratory, it was thought important to repeat those studies, using the immunodiffusion kit, in mice infected with both influenza A and B viruses. Once it had become established when the protein achieved maximal levels in relation to lung consolidation, lung virus titres, SaO2 decline, and occurrence of death, experiments were run comparing serum AGP levels to these other disease parameters in mice treated with the influenza neuraminidase inhibitor oseltamivir, which has been shown to be markedly effective in the treatment of murine influenza infections (Mendel et al., 1998; Sidwell et al., 1998), or with the broad spectrum antiviral ribavirin (Sidwell et al., 1972).

Materials and methods

Animals
Female specific pathogen-free 18-21 g BALB/c mice were obtained from B & K International (Fremont, Calif., USA). Housing and care of the animals were as described previously (Sidwell et al., 1992).

Viruses
Influenza A/Shangdong/09/93 (H3N2) was provided by H Regnery (Centers for Disease Control and Prevention, Atlanta, Ga., USA). It was passaged seven times through mice and then a pool was prepared in MDCK cells. Influenza B/Hong Kong/05/72 virus was obtained from the American Type Culture Collection (Manassas, Va., USA). It was passaged a single time through mice and used as a mouse lung homogenate. Both virus pools were stored at –80°C and pretitrated in mice before use in this study.
Serum alpha-1-acid glycoprotein as parameter for in vivo assay

Alpha-1-acid glycoprotein determination
Serum was assayed by single radial immunodiffusion using a kit from Saikin Kagaku Institute (Sendai, Japan). The kits contained test plates with 10 wells each. Standard solutions of mouse AGP at 1000 µg/ml and 250 µg/ml were included in the kits as controls.

SaO2 determination
An Ohmeda Biox 3740 pulse oximeter (Ohmeda, Louisville, OH, USA) was used to measure percentage SaO2 in the mice. The human ear probe attachment was placed on the thigh of the animal, with readings made after a 20 s stabilization time on each animal as previously described (Sidwell et al., 1992).

Lung virus titre determination
Each lung was homogenized to a 10% w/v suspension in minimum essential medium supplemented with 0.18% NaHCO3 and 50 µg gentamicin/ml. Each homogenate was assayed in triplicate in MDCK cells in 96-well microplates with viral cytopathic effect determined visually as an endpoint, as described previously (Sidwell et al., 1985).

Test compound
Oseltamivir was provided by Dr Chung Kim of Gilead Sciences (Foster City, Calif., USA). Ribavirin was obtained from ICN Pharmaceuticals (Costa Mesa, Calif., USA). Each was dissolved in sterile saline for use in these experiments.

Statistical analysis
Increases in survivor numbers were evaluated by χ2 analysis with Yates’ correction for small sample size. Differences in mean day to death, SaO2 levels, virus titres, and AGP levels were analysed by t test. Wilcoxon ranked sum analysis was used for comparing differences in mean lung scores.

Experiment design: comparison of serum alpha-1-acid glycoprotein to influenza disease parameters
Experiments were done in mice infected with influenza A and B viruses to determine the rate of development of serum AGP, and to compare this rate with the development of the infection in the mice. In each experiment, a group of 50 mice were infected intranasally as described previously (Sidwell et al., 1998). For influenza A/Shangdong, this was a virus dose of $10^{5.5}$ 50% cell culture infectious doses (CCID50)/ml; for influenza B/Hong Kong, the virus dose was $10^{7.0}$ CCID50/ml. Five mice were killed on days 1, 3, 6, 9, 12 and 15 after virus exposure. The serum was assayed at each time point for AGP, and their lungs were assigned a consolidation score ranging from 0 (normal lung) to 4 (maximal plum coloration), weighed and assayed for virus.

Figure 2. Comparison of development of serum alpha-1-acid glycoprotein levels with disease parameters in mice infected with influenza B/Hong Kong/05/72 virus

(a) Arterial oxygen saturation, SaO2. (b) Lung scores. (c) Lung weights. (d) Lung virus titres.
Mice infected with a LD_{100} dose of influenza A (10^{5.0} CCID_{50}/ml) or B (10^{7.0} CCID_{50}/ml) virus were treated by oral gavage with oseltamivir at doses of 100, 10 or 1 mg/kg per day or with 75 mg/kg per day of ribavirin. Fifteen mice were used in each dosage group, with 30 infected animals per day or with 75 mg/kg per day of ribavirin. Fifteen mice were used in each dosage group, with 30 infected animals treated in parallel with sterile H_{2}O. Ten animals in each group were killed on day 6 and their lungs removed, assigned a consolidation score, weighed and assayed for lung virus titre. Their serum was also taken on day 6 and assayed for AGP titre.

As controls, 35 uninfected mice were run in parallel in each experiment; five were killed at the same time as the infected mice and the same parameters described above determined to provide baseline data. The remaining five mice were assayed for SaO_{2} levels in parallel with the infected animals.

**Table 1. Effect of orally administered oseltamivir or ribavirin on an influenza A virus infection in mice:**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/day)</th>
<th>Survival</th>
<th>MDD§</th>
<th>Day 10 SaO_{2}</th>
<th>Lung score (mg/ml)</th>
<th>Lung weight (% of normal)</th>
<th>Lung virus titre (log_{10}/g)</th>
<th>AGP titre (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oseltamivir</strong></td>
<td>100</td>
<td>10/10</td>
<td>21.0 ±0.0</td>
<td>10.5 ±2.4</td>
<td>105.2 ±4.7</td>
<td>21.0 ±1.3</td>
<td>180 ±26</td>
<td>88.6 ±2.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9/10</td>
<td>10.0 ±0.0</td>
<td>10.5 ±2.4</td>
<td>105.2 ±4.7</td>
<td>21.0 ±1.3</td>
<td>180 ±26</td>
<td>88.6 ±2.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4/5</td>
<td>12.2 ±3.3</td>
<td>10.5 ±2.4</td>
<td>105.2 ±4.7</td>
<td>21.0 ±1.3</td>
<td>180 ±26</td>
<td>88.6 ±2.6</td>
</tr>
<tr>
<td><strong>Ribavirin</strong></td>
<td>75</td>
<td>10/10</td>
<td>21.0 ±0.0</td>
<td>10.5 ±2.4</td>
<td>105.2 ±4.7</td>
<td>21.0 ±1.3</td>
<td>180 ±26</td>
<td>88.6 ±2.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9/10</td>
<td>10.0 ±0.0</td>
<td>10.5 ±2.4</td>
<td>105.2 ±4.7</td>
<td>21.0 ±1.3</td>
<td>180 ±26</td>
<td>88.6 ±2.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3/10</td>
<td>12.2 ±3.3</td>
<td>10.5 ±2.4</td>
<td>105.2 ±4.7</td>
<td>21.0 ±1.3</td>
<td>180 ±26</td>
<td>88.6 ±2.6</td>
</tr>
</tbody>
</table>

‡ Mean day to death of mice dying before day 21.
*Mean day to death of mice dying before day 21.
† Alpha-1-acid glycoprotein measured by single radial immunodiffusion.

**Experiment design: antiviral studies**

Mice infected with a LD_{100} dose of influenza A (10^{10} CCID_{50}/ml) or B (10^{10} CCID_{50}/ml) virus were treated by oral gavage with oseltamivir at doses of 100, 10 or 1 mg/kg per day or with 75 mg/kg per day of ribavirin. Fifteen mice were used in each dosage group, with 30 infected animals treated in parallel with sterile H_{2}O. Ten animals in each group were killed on day 6 and their lungs removed, assigned a consolidation score, weighed and assayed for lung virus titre. Their serum was also taken on day 6 and assayed for AGP titre. Serum taken at this time was analysed for levels of AGP. A portion of each lung was placed in formalin, sections made, stained with haematoxylin and eosin, and examined microscopically for abnormalities. A group of five infected mice were held the duration of the study for comparison of serum alpha-1-acid glycoprotein with other disease parameters.

**Results**

Comparison of serum alpha-1-acid glycoprotein levels to influenza disease parameters

The results of the experiment using influenza A virus infected mice are shown in Figure 1. This influenza A infection was lethal to 90% of the mice, their mean day to death being 6.5 days. The mean (±50) AGP level in uninfected mice was 74 (±16) µg/ml, in the infected mice, the protein rose rapidly to a maximum mean of 725 (±25) µg/ml by day 3, then gradually declined to near-normal levels by day 11. The SaO_{2} levels by day 3, then gradually declined to near-normal levels by day 11. The SaO_{2} levels (Figure 1a) began to significantly decline on day 6, reaching minimal levels of approximately 76% by day 9. Lung consolidation, as seen by lung scores of 1 or more, and mean weight increases of nearly double the normal weight, were also seen by day 3, although reached maximal consolidation by day 5 (Figures 1b, c). Lung virus titres rose dramatically to maximal levels of approximately 10^{7} CCID_{50}/g by day 1 (Figure 3d).

The data obtained using influenza B virus-infected mice are seen in Figure 2. This infection was less lethal to the mice, killing only 10%, but the AGP levels rose at about the same rate as seen in the influenza A virus-infected mice, reaching a mean maximum level of 565 (±75) µg/ml by day 3, gradually declining thereafter (Figure 2), with less lethality, the SaO_{2} decline was less pronounced, but occurred beginning on day 6 (Figure 2a). Lung consolidation, as shown by increased lung scores and lung weights, was significantly increased in the mice by day 3, correlating...
well with AGP levels (Figures 2h, c). High lung virus titres were seen in the mice with the maximal titres seen at about the same time as the AGP levels (Figure 2d).

Histological analysis of lungs from each infected group revealed a typical pattern of influenza virus induced lung inflammation. On day 1, thickening of alveolar walls accompanied by about 10% of the bronchial epithelial cells having pyknotic and karyorrhectic nuclei indicative of necrosis was seen. This occurred on the same day lung virus titres were at their peak and AGP levels were significantly increasing. By day 3, the same anomalies were seen to a greater extent accompanied by macrophages, lymphocytes and neutrophils in the alveolar spaces. As the infection progressed in the animal, changes in the lung indicating interstitial pneumonia became more pronounced. Importantly, the initial damage to lung tissue indicative of inflammation occurred when the AGP values were at their maximum.

Antiviral experiments

The results of the experiment using oral gavage administered oseltamivir or ribavirin on an influenza B virus infection in mice are summarized in Table 3. As expected, based on earlier studies done with these compounds, significant antiviral efficacy was observed using each, with the effects of oseltamivir being dose responsive by all parameters. The antiviral effect was also seen in significantly lowered serum AGP and high AGP levels together with mild SaO₂ decline occurred. Therapy with 20 mg/kg per day of oseltamivir was used. As expected, the disease induced in the mice increased with viral challenge dose, the highest (10⁻³ dilution, 10⁻⁴ CCID₅₀/ml) challenge being 100% lethal to the mice, with the animals having a mean day to death of only 4.9 (±0.7) days. The 10⁻⁴ viral challenge was only lethal to 50% of the animals, and the 10⁻⁵ viral challenge did not kill the mice, although lung consolidation, high virus titres in the lungs, and high AGP levels together with mild SaO₂ decline occurred. Therapy with 20 mg/kg per day of oseltamivir was significantly inhibitory at all viral challenge doses. This antiviral effect was also seen in significantly lowered serum AGP levels in all groups.

Discussion

These data show that mouse serum levels of AGP, as determined by a commercially available kit, increase significantly following infection with either influenza A or B. These results confirm and extend the findings of Wong et al. (1989). The data clearly show that a rise in AGP occurs in influenza A viral challenge dose on the antiviral efficacy of oseltamivir (Table 3). Again, the effects of the disease parameters were compared with the serum AGP levels. In this study, a single dose, 20 mg/kg per day of oseltamivir was used. As expected, the disease induced in the mice increased with viral challenge dose, the highest (10⁻³ dilution, 10⁻⁴ CCID₅₀/ml) challenge being 100% lethal to the mice, with the animals having a mean day to death of only 4.9 (±0.7) days. The 10⁻⁴ viral challenge was only lethal to 50% of the animals, and the 10⁻⁵ viral challenge did not kill the mice, although lung consolidation, high virus titres in the lungs, and high AGP levels together with mild SaO₂ decline occurred. Therapy with 20 mg/kg per day of oseltamivir was significantly inhibitory at all viral challenge doses. This antiviral effect was also seen in significantly lowered serum AGP levels in all groups.

A comparison of influenza disease parameters and serum AGP levels indicate the closest correlation to exist between
lung virus titres, consequent lung inflammation, and the protein levels. Since AGP is an acute phase protein, which has previously been shown to respond to inflammation (McGregor et al., 1987), such correlation would be expected. The macrophage is most commonly associated with initiating the cascade of events occurring in the acute phase response (Baumann & Goukie, 1994), and these cells were seen in abundance by day 3, when the AGP levels reached maximal levels. A primary objective in conducting these experiments was to determine if AGP levels could be used as an additional parameter for evaluating the reduction of viral-induced lung tissue damage by potential antiviral drugs in the mouse model. With both influenza A and B, the levels of this protein were reduced by antiviral therapy and correlated well with other influenza disease parameters. Therapy with both oseltamivir, a selective inhibitor of influenza virus neuraminidase (Mendel et al., 1998) and ribavirin, whose triphosphate inhibits influenza virus RNA polymerase (Eriksson et al., 1977) and more generally blocks DNA synthesis via inhibition of IMP dehydrogenase (Streeter et al., 1979), inhibited the elevation of serum AGP in the infected animals. It was noted, however, that this inhibition was less in the influenza B virus-infected mice than in those infected with influenza A virus. This lesser effect may have been a reflection of the viral challenge, since the influenza B virus-infected mice had a mean day to death of 8.0 days compared with 10.5 days in the influenza A virus-infected mice. In the antiviral experiment with influenza A the serum AGP levels, while elevated, were not at the maximal levels seen in Figure 1. This was primarily due to the time (day) on which the animals were sacrificed in the antiviral experiment, which was after the maximal levels occurred in the kinetic experiment. This time was selected in order to obtain greater lung consolidation and more SaO2 decline. In the influenza B-virus antiviral experiment, the day 5 AGP levels were higher than those seen in the initial kinetics study. This was presumably due to the higher virus challenge dose used in the antiviral study. Although an earlier time of assay would be preferable for measurement of serum AGP if lung parameters are also to be evaluated, then the day 5 time of sacrifice would be recommended in future studies since at this latter time all parameters were significantly elevated.

These experiments indicate that measurement of serum AGP levels may be used to monitor anti-influenza therapy in the mouse model, and may be of particular value when viral challenge doses are relatively low, resulting in low lethality in the mouse. This was especially illustrated in the present study, in which mice were challenged with varying concentrations of virus.

Acknowledgements

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References


Table 3. Influence of influenza A virus challenge dose on the efficacy of orally administered oseltamivir: comparison of serum AGP with other disease parameters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viral challenge (ECD50/ml)</th>
<th>Survival (days alive)</th>
<th>Lung score</th>
<th>Lung weight (log10 mg)</th>
<th>Lung virus titre (log10 cp/ml)</th>
<th>AGP titre µg/ml ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oseltamivir 10</td>
<td>8/8</td>
<td>21.0 ±0.0</td>
<td>88.1 ±1.8ε</td>
<td>5.3 ±0.5α</td>
<td>152 ±10β</td>
<td>5.1 ±0.4±11</td>
</tr>
<tr>
<td>H2O</td>
<td>4/8</td>
<td>8.0 ±2.4</td>
<td>80.5 ±6.0</td>
<td>6.8 ±0.5</td>
<td>361 ±19</td>
<td>361 ±19</td>
</tr>
<tr>
<td>Oseltamivir 20</td>
<td>8/8</td>
<td>21.0 ±0.0</td>
<td>87.1 ±1.2ε</td>
<td>5.3 ±0.5α</td>
<td>165 ±24β</td>
<td>5.3 ±0.5±11</td>
</tr>
<tr>
<td>H2O</td>
<td>4/8</td>
<td>8.0 ±2.4</td>
<td>81.0 ±6.0</td>
<td>6.8 ±0.5</td>
<td>361 ±19</td>
<td>361 ±19</td>
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<tr>
<td>Oseltamivir 30</td>
<td>8/8</td>
<td>21.0 ±0.0</td>
<td>87.1 ±1.5α</td>
<td>5.3 ±0.5α</td>
<td>165 ±24β</td>
<td>5.3 ±0.5±11</td>
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<td>361 ±19</td>
</tr>
</tbody>
</table>

Oseltamivir was administered orally at 20 mg/kg per day, twice daily ×5, beginning 4 h before virus exposure. *P<0.05, †P<0.01, ‡P<0.001 compared with appropriate H2O-treated controls. §Mid day to death of mice dying before day 21. Arterial oxygen saturation.
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