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

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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 (SaO₂), 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, SaO₂ 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

Introduction

Alpha-1-acid glycoprotein (AGP) is one of the major acute phase proteins in serum produced by macrophages, liver cells, and polymorphonuclear lymphocytes (Schmid, 1975). The material increases dramatically in mammalian systems when inflammation, pregnancy, or cancer occurs (Buschus, 1975; Schmid, 1975). The protein has also been referred to as an immunosuppressive acidic protein, since it displays suppressive activity against various immune responses in vitro and in vivo (Tamura et al., 1981; Aso et al., 1992). Virus infections have been shown to cause an increase in the serum concentration of two acute phase reactants (C-reactive protein and amyloid A protein) (Salonen & Vaheri, 1981; Griffin et al., 1983; Whicher et al., 1985), suggesting that AGP may respond similarly. Wong et al. (1989) have subsequently reported that acute influenza A virus infections in mice result in a moderate (up to 48%) increase in this protein, finding a correlation with AGP serum level and lung consolidation.

As a follow-up to that latter report, it was thought that the increased AGP seen in the serum of influenza virus infected mice may be useful as an additional parameter for the evaluation of the efficacy of potential antiviral drugs. Current methods of assessing influenza disease progression are not always accurate. The assignment of a score for lung consolidation is quite subjective; lung weight increase is a general assessment that indicates increased...
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 antiserum 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 reinoculated in mice before use in this study.

Figure 1. Comparison of development of serum alpha-1-acid glycoprotein levels with disease parameters in mice infected with influenza A/Shangdong/09/93 (H3N2) virus

(a) Arterial oxygen saturation, SaO₂. (b) Lung scores. (c) Lung weights. (d) Lung virus titres.
AGP, alpha-1-acid glycoprotein.
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 kit 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 \( \chi^2 \) 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. AGP, alpha-1-acid glycoprotein.
Drug-treated group and 20 H2O-treated controls were assayed from days 3 to 11 for levels of SaO2; deaths were noted daily for 21 days, and SaO2 values determined for death for 21 days, and SaO2 values determined daily from days 3 to 11, during the time period when SaO2 decline usually occurs (Shibell et al., 1992).

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 were determined to provide baseline data. The remaining five mice were assayed for SaO2 levels in parallel with the infected animals.

**Experiment design: antiviral studies**

Mice infected with a LD100 dose of influenza A (105.0 CCID50/ml) or B (107.0 CCID50/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 H2O. Ten animals in each group were killed on day 6 and their lungs removed, assigned a consolidation score, weighed and assayed for virus titre. Their serum was also taken on day 6 and assayed for SaO2 levels to influenza disease parameters.

**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 3. This influenza A infection was lethal to 90% of the mice, their mean day to death being 6.5 days. The mean (±SD) AGP level in uninfection 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 SaO2 levels (Figure 1a) began to significantly decline on day 6, reaching minimum levels of 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 109 CCID50/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 this less lethal infection, the SaO2 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 with the increased viral titres and SaO2 levels.
Table 2. Effect of orally administered oseltamivir or ribavirin on an influenza B virus infection in mice: comparison of serum alpha-1-acid glycoprotein with other disease parameters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg/day)</th>
<th>Survival</th>
<th>MDD§ (days ± SD)</th>
<th>Day 10 SaO2* (µl/ml ± SD)</th>
<th>Lung score (mg/µl)</th>
<th>Lung weight (µg/µl)</th>
<th>Lung virus titre (log10/g ±SD)</th>
<th>AGP¶ titre (µg/ml ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oseltamivir</td>
<td>100</td>
<td>8/9*</td>
<td>13.0 ±0.0</td>
<td>85.4 ±2.4*</td>
<td>0.5 ±0.1</td>
<td>180 ±17</td>
<td>3.5 ±1.2</td>
<td>311 ±27*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2/10</td>
<td>9.6 ±0.0*</td>
<td>77.7 ±4.4*</td>
<td>1.0 ±0.0</td>
<td>164 ±9</td>
<td>3.8 ±0.5</td>
<td>321 ±54*</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3/10*</td>
<td>7.3 ±2.8*</td>
<td>79.5 ±8.1</td>
<td>2.1 ±0.4</td>
<td>250 ±43</td>
<td>3.6 ±0.1</td>
<td>290 ±26*</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>75</td>
<td>7/9*</td>
<td>8.0 ±0.0</td>
<td>84.1 ±14</td>
<td>0.8 ±0.2</td>
<td>152 ±41</td>
<td>3.1 ±0.5</td>
<td>415 ±109</td>
</tr>
<tr>
<td>H2O –</td>
<td>0/17</td>
<td>6.0 ±1.1</td>
<td>75.0 ±0.0</td>
<td>2.8 ±1.0</td>
<td>284 ±60</td>
<td>3.9 ±0.5</td>
<td>512 ±139</td>
<td></td>
</tr>
</tbody>
</table>

Oseltamivir was administered twice daily, ×, beginning 4 h before virus exposure.

*P<0.05, ‡P<0.01, ¶P<0.001 compared with H2O-treated controls.

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 mice infected with either influenza A or B viruses, and that the degree of AGP elevation appears dependent on viral challenge dose, and thus is a reflection of tissue damage due to the virus infection. A comparison of influenza disease parameters and serum AGP levels indicates the closest correlation to exist between

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 animals, 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 maxima.

Antiviral experiments

The results of the experiment using oral gavage administered oseltamivir or ribavirin on an influenza A virus infection in mice are summarized in Table 1. As expected, based on earlier studies done with these compounds, significant antiviral efficacy was observed with each, with the effects of oseltamivir being dose responsive by all parameters. The antiviral effect was also seen in significantly lowered serum AGP levels in all groups. This antiviral effect was also seen in significantly lowered serum AGP levels in all groups.
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>Virus challenge</th>
<th>Survival</th>
<th>MD50</th>
<th>Lung score</th>
<th>Lung weight</th>
<th>Lung virus titre</th>
<th>AGP titre</th>
<th>Day 5 mean data</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CCID50/ml)</td>
<td>(days)</td>
<td>(%)</td>
<td>(mg)</td>
<td>(log10/g)</td>
<td>(g/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/8</td>
<td>8.0 ±2.4</td>
<td>80.5 ±6.0</td>
<td>2.8 ±0.9</td>
<td>360 ±58</td>
<td>6.1 ±0.4</td>
<td>361 ±107</td>
<td></td>
</tr>
<tr>
<td>4/8</td>
<td>8.0 ±2.4</td>
<td>86.9 ±1.6</td>
<td>0.0 ±0.0</td>
<td>152 ±10</td>
<td>5.1 ±0.4</td>
<td>110 ±29</td>
<td></td>
</tr>
<tr>
<td>0/8</td>
<td>4.9 ±0.7</td>
<td>75.0 ±0.0</td>
<td>3.5 ±0.6</td>
<td>435 ±50</td>
<td>6.8 ±0.5</td>
<td>435 ±19</td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>0.8*</td>
<td>0.8*</td>
<td>0.8*</td>
<td>0.8*</td>
<td>0.8*</td>
<td>0.8*</td>
<td>0.8*</td>
</tr>
<tr>
<td>Oseltamivir 10</td>
<td>8/8</td>
<td>8.8 ±1.2</td>
<td>87.1 ±5.1</td>
<td>165 ±24</td>
<td>5.3 ±0.5</td>
<td>121 ±41</td>
<td></td>
</tr>
<tr>
<td>Oseltamivir 3.75</td>
<td>8/8</td>
<td>8.0 ±2.0</td>
<td>87.1 ±2.0</td>
<td>165 ±19</td>
<td>5.1 ±0.5</td>
<td>130 ±16</td>
<td></td>
</tr>
<tr>
<td>Oseltamivir 4.75</td>
<td>8/8</td>
<td>7.8 ±3.0</td>
<td>76.0 ±2.8</td>
<td>435 ±19</td>
<td>5.1 ±0.7</td>
<td>223 ±29</td>
<td></td>
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<tr>
<td>Oseltamivir 5.75</td>
<td>8/8</td>
<td>7.0 ±2.0</td>
<td>87.1 ±1.0</td>
<td>165 ±24</td>
<td>5.3 ±0.5</td>
<td>121 ±41</td>
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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. *Mean day to death of mice dying before day 21.

(Arterial oxygen saturation.)

*Alpha-1-acid glycoprotein measured by single radial immunodiffusion.

lung virus titres, concomitant 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 & Goukle, 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 (Ericsson et al., 1977) and more generally blocks DNA synthesis via inhibition of IMP dehydrogenase (Streeter et al., 1973), Drabikowski 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 6.0 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 study. 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.

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References


Serum alpha-1-acid glycoprotein as parameter for in vivo assay


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