The Significance of Influenza Virus Neuraminidase in Immunity

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SUMMARY

The neuraminidase of the Rostock strain of fowl plague virus (FPV) was found to be serologically closely related to the enzyme of swine influenza virus, whereas the haemagglutinins were distinct. When chickens were immunized with the apathogenic swine influenza virus, they were fully protected against an infection with the highly pathogenic FPV which killed non-immunized birds within 2 days. These experiments provide conclusive evidence that a protective capacity can also be attributed to the neuraminidase of influenza viruses.

INTRODUCTION

The detection of haemagglutinin and neuraminidase as two antigenically distinct entities of the influenza virus envelope (Drzeniek, Seto & Rott, 1966), which can be independently exchanged by recombination (Laver & Kilbourne, 1966), facilitated the antigenic analysis and differentiation of the multitude of strains of influenza A viruses. When monospecific antisera became available, a decisive difference between antibodies directed against the two envelope antigens was apparent. While anti-haemagglutinin serum neutralized virus infectivity, antibodies to neuraminidase had no influence on infectivity, but in their presence virus release was inhibited (Seto & Rott, 1966).

A variety of cross-reactivities are known among the haemagglutinins and neuraminidases, not only of mammalian strains, but also among mammalian and avian influenza A strains (Webster, 1972).

We describe in this report a close antigenic similarity between the neuraminidases of swine influenza and the Rostock strain of fowl plague virus (FPV) – both these viruses having different haemagglutinins. These antigenic properties of the virus envelope could be used to reassess the question of the immunogenic potency of neuraminidase in the chicken, for which Allan, Madeley & Kendal (1971) had presented some evidence. In our experiments immunity could be induced with the non-pathogenic swine influenza virus, and this immunity was challenged with the highly pathogenic FPV.

METHODS

Viruses. Fowl plague virus (FPV/Rostock/34; Traub, 1942), A/swine/1976/31 was obtained from Dr Davenport, Ann Arbor (swine Ann Arbor), A/swine/1976/31 was obtained from Dr Drescher, Hannover (swine Hannover), A/swine/Shope/31 was obtained from Dr Werner, Berlin (swine Berlin), A/swine/Iowa/30 was obtained from Dr Schild, London (swine London) and virus N (Rott & Schäfer, 1960). All viruses were grown in the allantoic cavity of 11-day-old chick embryos. Infectious allantoic fluids were harvested aseptically, titrated by haemagglutination and used as such for injecting the experimental chickens.
Immunization procedure. Series 1 consisted of 20 chickens, about 15 weeks old, which were injected intraperitoneally with 2 ml of allantoic fluid containing swine Ann Arbor (haemagglutination (HA) titre 1/256), four times at intervals of 3 to 4 days. Three days after the last injection 4 groups were formed which were challenged by intramuscular injections with 500, 1000, 5000 and 10000 p.f.u. of FPV, respectively. Five control birds received 500 p.f.u. simultaneously.

Series 2 consisted of 5 chickens which were inoculated once intravenously with 2 ml allantoic fluid containing swine Ann Arbor (HA titre 1/512), and 2 weeks later these animals and 2 control birds were inoculated intramuscularly with 1000 p.f.u. of FPV.

Blood samples were obtained by cardiac puncture at intervals of 3 to 4 days. The sera were inactivated at 56 °C for 30 min.

For haemagglutination-inhibition tests serum samples were treated with M/9 o periodate (Davenport, Rott & Schafer, 1960). Eight H.A.U. of each virus were used.

Enzyme antibody titres were measured as described previously (Drzeniek et al. 1966). Lyophilized fetuin, dissolved in 0.15 M-phosphate buffer, pH 7.2, equivalent to a concentration of 50 µg neuraminic acid/0.1 ml, was used as substrate. Equal vol. of neuraminidase liberating 6 to 8 µg neuraminic acid in 30 min at 37 °C and serum dilutions were mixed, 0.1 ml of fetuin was added to the reaction tubes, and the enzyme reaction was kept at 37 °C for 30 min. Antibody titre is expressed as that dilution which inhibited 50 % of the enzyme activity.

Antibodies against purified neuraminidase were prepared by injecting rabbits with the enzyme obtained from Tween/ether disrupted virus which had been treated with pronase and purified by sucrose density centrifuging (Seto, Drzeniek & Rott, 1966; Becht, Hämmerling & Rott, 1971).

Plaque tests were carried out as described by Zimmermann & Schafer (1960).

Antibodies against the ribonucleoprotein (RNP) antigen were assayed by an indirect haemagglutination procedure essentially as described previously (Becht, 1968).

Influence of antisera on virus yields. Heat-inactivated swine Ann Arbor antiserum, with a final neuraminidase inhibition titre of 1/200, was added in the medium to chick embryo fibroblasts infected with FPV or swine Ann Arbor immediately after the adsorption period (30 min). Haemagglutinating activity and p.f.u. of medium and cells were tested 8 h after infection.

For testing the influence of anti-neuraminidase antibodies on the size and numbers of plaques, antiserum at the same final dilutions was incorporated into the melted agar (Seto & Rott, 1966).

RESULTS

Antigenic similarity between neuraminidases from FPV and swine influenza

Antisera directed against FPV inhibited neuraminidase activity of 4 strains of swine influenza (Table 1). This held true for sera from rabbits immunized with purified enzyme and for sera from animals injected with whole virus. Serum from a chicken that had survived a FPV infection inhibited swine neuraminidase as efficiently as did rabbit hyperimmune sera.

Conversely, serum from chickens injected with swine influenza virus inhibited FPV neuraminidase almost as efficiently as the enzyme associated with the swine strains. The heterologous titres of enzyme inhibition reactions between FPV and swine influenza were only slightly lower than the respective homologous titres, and the cross-reactions were rather symmetric with the exception of the London strain of swine influenza. Serum from
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Table 1. **Neuraminidase inhibition tests comparing different strains of swine influenza virus with FPV**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Virus</th>
<th>Swine Ann Arbor</th>
<th>Swine Hannover</th>
<th>Swine Berlin</th>
<th>Swine London</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken anti-FPV</td>
<td>FPV</td>
<td>100*</td>
<td>30</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Rabbit anti-FPV</td>
<td>400</td>
<td>100</td>
<td>90</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>Rabbit anti-FPV neuraminidase</td>
<td>100</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Chicken anti-swine Ann Arbor</td>
<td>100</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Chicken anti-swine London</td>
<td>&lt; 10</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Titres are expressed as the reciprocal of the antibody dilutions inhibiting 50% of the enzyme activity.

Table 2. **Haemagglutination inhibition tests with different strains of swine influenza virus and FPV**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Virus</th>
<th>Swine Ann Arbor</th>
<th>Swine Hannover</th>
<th>Swine Berlin</th>
<th>Swine London</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken anti-FPV</td>
<td>256*</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
</tr>
<tr>
<td>Rabbit anti-FPV</td>
<td>128</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
<td>&lt; 8</td>
</tr>
<tr>
<td>Chicken anti-swine Ann Arbor</td>
<td>&lt; 8</td>
<td>4000</td>
<td>4000</td>
<td>4000</td>
<td>2000</td>
</tr>
<tr>
<td>Chicken anti-swine London</td>
<td>&lt; 8</td>
<td>500</td>
<td>500</td>
<td>1000</td>
<td>500</td>
</tr>
</tbody>
</table>

* Titres are expressed as the reciprocal of the antibody dilutions showing a complete inhibition of 8 H.A.U.

Table 3. **Neutralization of FPV with swine influenza antiserum**

<table>
<thead>
<tr>
<th>Serum</th>
<th>FPV (p.f.u./ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken normal</td>
<td>31</td>
</tr>
<tr>
<td>Chicken anti-swine Ann Arbor</td>
<td>15</td>
</tr>
</tbody>
</table>

* Expressed as the p.f.u./ml, the mean of 4 replicates.

The antiserum used was produced in chicken as described in the text. The titre for inhibition of swine Ann Arbor NA was 1:2000, and the HA inhibition titre was 1:4000. A 1:5 dilution of this antiserum was mixed with an equal part of PBS containing 50 to 100 p.f.u./ml of FPV. After 30 min at room temperature the mixture was assayed by the plaque test.

A chicken immunized with this strain did not inhibit FPV neuraminidase significantly, but anti-FPV sera blocked neuraminidase activity of this swine influenza strain (Table 1).

Haemagglutination of FPV and the swine strains, on the other hand, was inhibited only by antisera directed against their respective homologous haemagglutinins (Table 2).

Since it had been established that anti-neuraminidase serum does not neutralize infectivity but interferes with virus release, anti-swine serum should not neutralize an infection with FPV, but would be expected to impair the liberation of virus particles from the cells.

Results shown in Table 3 demonstrate that this was the case. Anti-swine serum did not prevent the infection of chick embryo fibroblasts with FPV significantly, but its presence in the culture medium prevented the release of any significant HA activity or infectivity...
Table 4. Influence of anti-swine influenza serum on the multiplication of FPV and swine influenza virus in chick embryo fibroblasts

<table>
<thead>
<tr>
<th>Serum</th>
<th>FPV Medium H.A.U./ml</th>
<th>FPV Cells p.f.u./ml</th>
<th>Swine Ann Arbor Medium H.A.U./ml</th>
<th>Swine Ann Arbor Cells p.f.u./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken normal</td>
<td>64</td>
<td>1.4 x 10^8</td>
<td>1000</td>
<td>3.3 x 10^8</td>
</tr>
<tr>
<td>Chicken anti-swine</td>
<td>&lt; 2</td>
<td>7 x 10^2</td>
<td>32</td>
<td>8 x 10^3</td>
</tr>
</tbody>
</table>

After infection of the cells with an input multiplicity of about 10 p.f.u. the cell layer was incubated with a medium containing anti-swine serum in a dilution inhibiting > 98% of neuraminidase activity. Eight h after infection medium and cells were harvested separately and assayed.

Fig. 1. Plaque production of FPV in the presence of anti-swine influenza serum. The agar overlay contained normal chicken serum (a) or anti-swine serum in a dilution inhibiting > 98% neuraminidase activity in the standard inhibition test (b).

Immunization of chickens against FPV with swine influenza virus

Eight h after the injection of allantoic fluid containing live swine influenza virus into chickens, the agent could be re-isolated from the blood of 2 out of 5 randomly selected birds. Virus could not be demonstrated in blood samples subsequently drawn. There is additional evidence that the virus replicated in the infected animals. HA-inhibiting antibodies appeared 4 days after infection and rose steadily until the 18th day. Antibodies inhibiting neuraminidase activity of swine influenza virus appeared at a similar rate (Fig. 2). When antibodies against the RNP-antigen were measured 7 days post-inoculation in a passive haemagglutination assay, high titres (1:1000) were found in the serum of all chickens.
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Fig. 2. Production of specific antibodies by chickens inoculated with swine influenza Ann Arbor and challenged with FPV. The titres represent arithmetic means of 5 randomly selected animals from the experimental group of 20. HA inhibition of swine Ann Arbor (○○○○○) and FPV (▲▲▲▲▲▲); 50 % inhibition of neuraminidase activity of swine Ann Arbor (××××××) and FPV (■■■■■■). The arrow indicates the time of challenge with FPV.

Challenge of the immunity induced by the swine virus with virulent FPV

All control animals which received 500 p.f.u., the lowest dose used, died the second day after inoculation. At autopsy they had the pathological symptoms typical for FPV infections.

Animals which had been immunized with swine influenza survived the challenge of FPV infection, without any clear clinical signs of fowl plague. This was true for four groups of animals challenged with 500, 1000, 5000 or 10000 p.f.u. of FPV simultaneously with the non-immunized control group. The birds appeared to be healthy 3 weeks later when they were killed.

After the challenge infection HA-inhibiting antibodies against FPV appeared in their serum, whereas HA-inhibiting antibodies of swine influenza declined (Fig. 2). A booster effect of neuraminidase inhibition of either the FPV or the swine influenza enzyme could be clearly seen 14 days after the challenge infection (Fig. 2). This finding stresses again the close similarity between the neuraminidases of the two strains in contrast to their distinct haemagglutinins.

Essentially identical results were obtained when one immunizing injection of swine influenza virus was administered intravenously. In this experiment all chickens survived a challenge with 1000 p.f.u. of FPV without any obvious clinical signs, whereas the non-immunized controls died the second day after infection.

Previous experience from this laboratory had shown that birds immunized with virus N, an avian influenza strain which is serologically unrelated to FPV (Rostock) (Rott & Schäfer, 1960), were unprotected against a challenge infection with FPV. In the course of these experiments a rooster had been immunized with virus N according to the schedule outlined in series 2 of Methods, except that a second immunizing injection had been applied 3 days after the first one. When this animal was challenged with 1000 p.f.u. of FPV (Rostock), it died 3 days later. A serum sample collected immediately before the challenging injection
2 weeks after the last immunizing dose inhibited haemagglutination by virus N at a dilution of 1:512 and 50% of the neuraminidase activity at a dilution of 1:150. This result is in agreement with the findings of Allan et al. (1971), who did not find any cross-protection among avian influenza strains which were unrelated in any one of the 2 envelope antigens.

**DISCUSSION**

Although the functional significance of the neuraminidase of myxoviruses still remains an open question, evidence has accumulated that this antigenic component of the virus envelope is of significance in contributing to the immune status of the host organism. Schulman, Khakpour & Kilbourne (1968) used recombinant viruses, possessing common neuraminidases and serologically unrelated haemagglutinins, and noted that anti-neuraminidase antibodies inhibited virus replication in the lungs of mice, diminishing the virus titre and the lung lesions. The common neuraminidase of influenza virus strains A2 and Hong Kong conferred considerable protection to mice vaccinated with influenza A2 and challenged with a mouse-adapted strain of Hong Kong (Schulman & Kilbourne, 1969). A similar role for the neuraminidase has been suggested to be of significance in man (Murphy, Kasel & Chanock, 1972). The clinical reactions of volunteers to an infection with a wild-type Hong Kong strain, the duration of virus excretion and the maximal level of virus shed were directly related to the level of anti-neuraminidase antibodies in their sera. Experiments carried out in a totally different system by Allan et al. (1971) showed that the similar neuraminidases of the 2 avian strains A/Turkey/England/63 and A/Tern/South Africa/61 were associated with partial protection of chicken against either one of the virulent viruses.

The lethal infection in avian systems offers the advantage that the efficiency of the immunization procedure can be clearly measured by their survival rates. When it was noted that the antigenicity of swine influenza neuraminidase was related to the Rostock strain of FPV, this provided a system to assess the immunogenic importance of the enzyme. All in vitro experiments showed that the swine strains examined had neuraminidases antigenically related with FPV (Rostock). Antisera against these viruses mutually inhibited neuraminidase activity. As expected from other experiments (Jahiel & Kilbourne, 1966; Seto & Rott, 1966), the addition of anti-swine influenza serum to the culture medium decreased the yield of FPV in the medium. The incorporation of this serum into the agar-overlay in plaque assays did not reduce the number of p.f.u., but it reduced plaque size, and it did not neutralize FPV infectivity for chick fibroblasts. The London strain of swine influenza, which showed an asymmetric cross-reactivity, is obviously a variant of this group, and therefore it was not used for immunization experiments. Such an exception in the rather homogeneous group of swine influenza viruses has been described (Meier-Ewert, Gibbs & Dimmock, 1970).

The close antigenic relationship among FPV (Rostock) and swine influenza virus neuraminidases has also been noted recently by other investigators (Tumova & Schild, 1972), and represents another example of common antigenic structures of mammalian and avian influenza strains besides the various cases already known (Pereira, 1969; Webster, 1972).

Immunization with swine influenza virus conferred a solid immunity against a highly excessive lethal dose of FPV. Swine influenza virus is particularly suited for these experiments, since it is not pathogenic for the chicken, but nevertheless undergoes a limited replication in the host. This was shown by the re-isolation of swine virus in 2 animals and, particularly, by the high titres of anti-RNP antibodies. The replication of the immunizing virus without provoking any clinical disturbances differs from the experimental conditions...
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employed by Allan et al. (1971) in which the pathogenic immunizing strains were inactivated by formalin. This is probably the reason that these authors only obtained partial protection, whereas the immunization procedure, used in our experiments, induced a solid immunity against a lethal dose of the challenge FPV.

Our results confirm observations in animal experiments and serological analysis in man that besides the haemagglutinin, the neuraminidase antigen has a definite significance as an immunogenic component of influenza viruses. This effect can be readily explained by the ability of the antibody to limit the spread of the virus in the host organism (Schulman et al. 1968) after virus particles had been entrapped in an antibody lattice at the surface of infected cells (Becht et al. 1971). However, since the virus constituent in question has been serologically defined by the inhibition of its enzymic activity with specific antibodies, the immunogenically active part of this virus envelope component might not be the same as the antigenic determinant of the active site of the enzyme and might involve a more complex structure.

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REFERENCES


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