Studies with Avian Influenza A Viruses: Cross Protection Experiments in Chickens

By W. H. ALLAN, C. R. MADELEY* and A. P. KENDAL†

Central Veterinary Laboratory, New Haw, Weybridge, Surrey, *Department of Clinical Virology, St Thomas's Hospital and Medical School, London S.E. 1 and †Department of Bacteriology, University College Hospital Medical School, London, W.C. 1

(Accepted 18 March 1971)

SUMMARY

Previous work on the serological relationships of avian influenza A viruses provided an opportunity to assess the protective effects of anti-neuraminidase antibody in a single species system. Three viruses naturally lethal for the chicken (A/DUTCH, A/TURKEY/ENGLAND/63 and A/TERN/SOUTH AFRICA/61) were selected so that two (A/DUTCH and A/TURKEY/ENGLAND/63) had an antigenically similar haemagglutinin, two (A/TURKEY/ENGLAND/63 and A/TERN/SOUTH AFRICA/61) had a similar neuraminidase and two (A/DUTCH and A/TERN/SOUTH AFRICA/61) were unrelated by surface antigens. Each virus was used to immunize a group of 7-week-old chickens using one dose of killed virus followed by one dose of live virus. Each group was then divided into three subgroups and each subgroup challenged with one of the viruses. The results of cross-challenge with live virus were assessed objectively on mortality. (1) Immunization with any of the viruses protected the birds completely against the homologous virus. (2) Immunization protected the birds completely against a heterologous virus possessing a similar haemagglutinin. (3) Immunization gave partial protection against a heterologous virus possessing a similar neuraminidase and this protection was significantly greater (P = 0.01) than unimmunized controls when birds immunized with A/TURKEY/ENGLAND/63 virus were challenged with A/TERN/SOUTH AFRICA/61. In birds immunized with A/TERN/SOUTH AFRICA/61 and challenged with A/TURKEY/ENGLAND/63 the protection was approaching significance (P < 0.1 > 0.05). The results of testing the sera from the immunized birds confirmed the serological relationships of the viruses.

INTRODUCTION

The exact roles of neuraminidase, and antibody to it, in infections due to influenza are still uncertain. Seto & Rott (1966) showed that the antibody, although it did not neutralize the virus, reduced the size of plaques in cell culture, and they suggested that the enzyme was concerned with virus release. Since then much evidence has been obtained in support of this view (Jahiel & Kilbourne, 1966; Webster & Laver, 1967; Kilbourne et al. 1968; Seto & Chang, 1969). These results were all obtained with in vitro systems. Schulman, Khakpour & Kilbourne (1968) showed that anti-neuraminidase antibody gave some protection to mice challenged with influenza, but the virus is not a natural pathogen for mice and their system was not homologous for one species.

* Present address: MRC Virus Research Unit, Institute of Virology, Glasgow, W.1.
† Present address: School of Public Health, University of Michigan, Ann Arbor, Michigan, U.S.A.
Our work with avian influenza viruses (Madeley, Allan & Kendal, 1971) gave us the opportunity to examine the role of neuraminidase in lethal infections using an entirely homologous system. By using virus grown in chick embryos in chickens which are naturally susceptible to the virus, the system was devoid of species barriers and the lethal nature of the infection in unprotected birds allowed an objective assessment of the result of challenge.

Three viruses were selected for this study so that two had antigenically similar haemagglutinins, two had similar neuraminidases and two had no known similar surface antigens. All three strains possessed the influenza A internal RNP group antigen. By immunization and cross-challenge it was possible, therefore, to estimate the protective effect of antibody to haemagglutinin and neuraminidase separately, and since the experiment was done under double-blind conditions, it was also possible to do a prospective study without subjective bias.

METHODS

Virus strains. The three viruses used were A/DUTCH, A/TURKEY/ENGLAND/63 and A/TERN/SOUTH AFRICA/61. Details of their origin and propagation were given by Madeley et al. (1971). A/DUTCH and A/TURKEY/ENGLAND/63 had a similar haemagglutinin. A/TURKEY/ENGLAND/63 and A/TERN/SOUTH AFRICA/61 had an antigenically similar neuraminidase. A/DUTCH and A/TERN/SOUTH AFRICA/61 were without known related surface antigens.

Haemagglutination-inhibition tests. These were done as described previously (Madeley et al. 1971).

Neuraminidase-inhibition tests. The automated procedure used was described by Kendal & Madeley (1969). For these tests it was modified slightly. The substrate was human serum glycoprotein (Cohn fraction IV-4) and it was buffered with 0.02 M-acetate containing 0.02 % sodium azide. Mixtures of enzyme (as whole virus) and substrate were incubated for 17 hr at 37° with serum at final dilutions of 1/50, 1/100, 1/200, 1/400 and 1/800. At the end of this incubation unused substrate and serum proteins were precipitated with 10 % trichloroacetic acid and removed by centrifugation. The supernatant fluids were then assayed for released N-acetyl neuraminic acid as described already. This modified procedure allowed serum dilutions as low as 1/10 to be used without encountering the non-specific inhibitions found previously at 1/50 (Kendal & Madeley, 1969; Madeley et al. 1971). Where 50 % inhibition was found at 1/50, these sera were then re-tested at 1/10, 1/20 and 1/40. Similar dilutions of a pool of normal chicken sera were used as controls.

Virus infectivity titrations were made in 9-day chick embryos from an isolated flock using seven eggs per dilution and fivefold dilution steps. The titre (in ELD 50) was estimated by the Spearman-Kärber method (Allan & Hebert, 1968).

Double-blind conditions. Virus was grown from seed by one of us and the infected allantoic fluids were then given code letters and labelled by someone not otherwise involved in the experiments. Coded virus was used to immunize birds and to propagate challenge virus in eggs. The birds were immunized and challenged and the results were assessed under code. At the same time the pre-challenge sera were assayed for haemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) antibody and all results obtained before the code was opened.

Immunization. One hundred and eighty 7-week-old Light Sussex x Buff crossbred chickens were divided into three groups of 60 birds. Each group of 60 birds was housed in a separate test unit with separate ventilation. While live virus was in use, each unit was serviced by a different attendant. The birds were individually wing-tagged for identification. Each virus was given to one group as an initial intramuscular injection of 0.5 ml./bird of undiluted
Avian influenza viruses

allantoic fluid treated with 1/1000 neutral formalin at 37° for 12 hr (equivalent to about 10⁵ ELD₅₀ before inactivation). This initial inactivated dose was necessary as the live viruses are all lethal for chickens. Fourteen days later a second injection of 2 × 10⁵ ELD₅₀ in 1 ml. of peptone broth diluent was given intramuscularly. After another 14 days, blood samples were taken from each bird immediately before challenge. No birds died as a result of immunization.

Challenge. Each group of 60 birds was divided into three subgroups of 20. They were rearranged so that each unit contained 20 birds immunized with each virus. Ten more birds of the same age were added to each unit as unimmunized controls. All 70 birds in each group were then challenged with live virus, using 2 × 10⁵ ELD₅₀ in 1 ml. peptone broth given intramuscularly. Each group was inspected daily and the deaths recorded.

Serological tests. The pre-challenge sera were pooled in lots of 20 for testing. Each pool of 20 birds corresponded with a challenge subgroup.

RESULTS

Haemagglutination-inhibition

Each virus was inhibited by homologous antibody and antibody to A/DUTCH and A/TURKEY/ENGLAND/63 cross-reacted with the other virus. Neither showed any cross-reaction with A/TERN/SOUTH AFRICA/61 (Table I).

Table 1. Haemagglutination-inhibition titres of sera from immunized birds

<table>
<thead>
<tr>
<th>Virus</th>
<th>Immune sera</th>
<th>A/DUTCH</th>
<th>A/TURKEY/ENGLAND/63</th>
<th>A/TERN/SOUTH AFRICA/61</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/DUTCH</td>
<td>1*</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>A/TURKEY/ENGLAND/63</td>
<td>64†</td>
<td>128</td>
<td>256</td>
<td>16</td>
</tr>
<tr>
<td>A/TERN/SOUTH AFRICA/61</td>
<td>64</td>
<td>32</td>
<td>64</td>
<td>128</td>
</tr>
</tbody>
</table>

* Pooled sera from 20 birds in challenge subgroups – see text for details.
† Highest dilution of serum giving complete inhibition of 8 H.A. units of virus. — titre of < 1/2. Normal sera gave no inhibition at 1/2.

Table 2. Neuraminidase-inhibition titres of sera from immunized birds

<table>
<thead>
<tr>
<th>Virus antigen</th>
<th>Immune sera</th>
<th>A/DUTCH</th>
<th>A/TURKEY/ENGLAND/63</th>
<th>A/TERN/SOUTH AFRICA/61</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/DUTCH</td>
<td>&gt; 800</td>
<td>&gt; 800</td>
<td>&gt; 800</td>
<td>20</td>
</tr>
<tr>
<td>A/TURKEY/ENGLAND/63</td>
<td>750</td>
<td>350</td>
<td>550</td>
<td>475</td>
</tr>
<tr>
<td>A/TERN/SOUTH AFRICA/61</td>
<td>725</td>
<td>125</td>
<td>675</td>
<td>&gt; 800</td>
</tr>
</tbody>
</table>

* Pooled sera from 20 birds in challenge subgroups – see text for details.
† Serum dilutions giving an observed 50 % reduction in virus enzyme activity. — titre of < 1/10. Normal sera gave no inhibition at 1/10.

Neuraminidase inhibition

A/TURKEY/ENGLAND/63 and A/TERN/SOUTH AFRICA/61 cross-inhibited, though one subgroup of A/TURKEY/ENGLAND/63 (No. 2) had a low titre of antibody against A/TERN/SOUTH
AFRICA/61 neuraminidase (Table 2). Fortunately this subgroup was challenged with its homologous virus and this unexpectedly low value did not affect the results of challenge with live virus. A/DUTCH neuraminidase was slightly inhibited (1/20) by one group of antisera to A/TURKEY/ENGLAND/63. This may have been due to slight interference with the test by HI antibody. Otherwise, A/DUTCH was not inhibited by antisera to either of the other viruses. The homologous titres were uniformly high.

Table 3. Summary of protection experiments

<table>
<thead>
<tr>
<th>Challenge virus</th>
<th>Immunizing virus</th>
<th>A/DUTCH</th>
<th>A/TURKEY/ENGLAND/63</th>
<th>A/TERN/SOUTH AFRICA/61</th>
<th>Unimmunized controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/DUTCH</td>
<td>A/DUTCH</td>
<td>19/19*</td>
<td>17/17</td>
<td>3/19</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 %†</td>
<td>100 %</td>
<td>16 %</td>
<td>0 %</td>
</tr>
<tr>
<td>A/TURKEY/ENGLAND/63</td>
<td>A/DUTCH</td>
<td>16/16</td>
<td>17/17</td>
<td>6/16</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 %</td>
<td>100 %</td>
<td>37 %</td>
<td>0 %</td>
</tr>
<tr>
<td>A/TERN/SOUTH AFRICA/61</td>
<td>A/DUTCH</td>
<td>0/16</td>
<td>11/18</td>
<td>16/16</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 %</td>
<td>61 %</td>
<td>100 %</td>
<td>0 %</td>
</tr>
</tbody>
</table>

* Number of birds surviving in group over total number.
† Percentage survival.

These results confirmed the possession by A/TURKEY/ENGLAND/63 and A/DUTCH of similar haemagglutinins and by A/TURKEY/ENGLAND/63 and A/TERN/SOUTH AFRICA/61 of similar neuraminidases. A/DUTCH and A/TERN/SOUTH AFRICA/61 showed no cross-reactions in these tests.

Challenge

None of the challenge subgroups finally consisted of 20 birds. During the experiment some birds were lost through feather picking and cannibalism following regrouping of the birds, and these were not included in the results. The results were analysed statistically, for which we are grateful to Miss Nancy Hebert, of the Central Veterinary Laboratory, Weybridge. The challenge viruses were fully lethal, as shown by the 100 % mortality in the controls, and immunization with the homologous virus protected fully (Table 3). Antibody to haemagglutinin (A/TURKEY/ENGLAND/63 v. A/DUTCH) was also associated with complete protection from the other virus with a similar haemagglutinin. Immunization with either A/DUTCH or A/TERN/SOUTH AFRICA/61 did not protect against the other. Though 3/19 birds immunized with A/TERN/SOUTH AFRICA/61 virus and challenged with A/DUTCH survived, this did not differ significantly from the control results ($P = 0.5$) and we have no evidence that the internal RNP antigen elicited any protective antibody. There was, however, considerable survival in the subgroup immunized with A/TURKEY/ENGLAND/63 and challenged with A/TERN/SOUTH AFRICA/61, and vice versa, where the viruses had similar neuraminidases. In the subgroup immunized with A/TURKEY/ENGLAND/63 and challenged with A/TERN/SOUTH AFRICA/61, the 11/18 survival rate was a highly significant improvement over the controls ($P = 0.01$). The converse group, where 6/16 survived, gave a result approaching significance ($P < 0.1 > 0.05$), and which might have been significant had it been possible to use larger groups of birds. Clearly, although anti-neuraminidase antibody was associated with some protection it was only partial in the face of the challenge used.
DISCUSSION

The serological results in this prospective study were as predicted, confirming our previous work (Madeley et al. 1971). There was reciprocal inhibition of haemagglutinin between A/DUTCH and A/TURKEY/ENGLAND/63, though the titres were slightly lower than before. No inhibition of haemagglutination by anti-neuraminidase antibody was observed (A/TURKEY/ENGLAND/63 v. A/TERN/SOUTH AFRICA/61), though high titres of antibody were present. The one-way cross-reaction between these viruses observed previously (Madeley et al. 1971) was not seen. The birds used in these experiments were older (7 weeks instead of 4½ weeks) and resisted the second live immunizing dose better. Consequently, there may have been less virus multiplication and less stimulus to produce antibody to minor surface components.

The challenge experiments were made in an entirely homologous system, virus grown in chick embryos was used to challenge chickens. These viruses cause a lethal infection in the chicken, thus providing a unique system as other influenza A viruses do not kill either their natural or other hosts except after adaptive passage. Such a system also allows an objective assessment of protection, as no subjective estimate of the degree of illness or pathogenic effect was involved. Though there was some sickness among the survivors of the challenge, we did not attempt to evaluate it for this reason.

The intramuscular challenge route was used for several reasons, despite the fact that it is not the normal route of infection. Firstly, an injection puts a known amount of virus into the chicken without loss by sneezing, coughing or exhalation. Elimination of some of such losses by anaesthesia was not practicable with the number of birds involved. Secondly, the viruses are pathogenic by injection, and this method brings the virus directly into contact with any serum antibody, though not with secretory antibody. The use of an aerosol, under standardized conditions, would have been preferable but, with several viruses and over 200 birds, this also was not practicable. On balance we preferred the artificial method rather than the natural one as being more certain. Nevertheless, this is a very severe challenge, probably more so than is natural exposure to the disease and it is necessary to consider the results of challenge with this in mind.

Antibody to haemagglutinin appeared to protect fully against challenge by virus possessing the homologous antigen; A/TURKEY/ENGLAND/63 protected against A/DUTCH and vice versa. With anti-neuraminidase antibody, the results could not be predicted. Schulman et al. (1968) reported reduction in the size of lung lesions in mice, both by actively induced and by passive antibody. In a lethal system such reduction might not be life-saving and it might in any case reflect only a reduced rate in the development of the lesions. In experiments in vitro anti-neuraminidase antibody reduces the size of plaques in cell cultures without reducing their number, but it is not known whether this is due to specific inhibition of the enzyme site (Jahiel & Kilbourne, 1966; Seto & Chang, 1969) or tethering of the released virus to the host cell (Compans, Dimmock & Meier-Ewert, 1969; Kendal & Madeley, 1970). Whatever the mechanism, such antibody might reduce the spread of virus both within the host and to other susceptible hosts (Schulman et al. 1968). Consequently, the results of cross-protection experiments involving neuraminidase only are very interesting.

The birds immunized with A/TURKEY/ENGLAND/63 and challenged with A/TERN/SOUTH AFRICA/61 showed a degree of protection significantly greater than that shown by the controls. In the converse experiment, the evidence of protection was almost significant and might have been so had we been able to use more birds.

Our results were obtained in an homologous system without species barriers. They are the most definite results yet obtained of the protective effect of antibodies to haemagglutinin.
and neuraminidase. In influenza A viruses from any species of origin known to date, haemagglutinin and neuraminidase undergo independent antigenic variation (Schulman & Kilbourne, 1969; Meier-Ewert, Gibbs & Dimmock, 1970; Madeley et al. 1971). It can therefore be inferred from these results that maximum protection would be provided by a vaccine containing the appropriate haemagglutinin, but antibodies induced by neuraminidase could provide some protection against new strains where only the haemagglutinin has changed. In terms of human disease it is interesting to note the comparative failure of influenza A2/HONG KONG/68 (new haemagglutinin, old neuraminidase, Coleman et al. 1968) to spread on its first appearance.

Transmission experiments were not attempted and therefore we cannot say if anti-neuraminidase antibody modifies the amount of virus released from an infected chicken. All the available evidence, however, suggests that this is likely.

The support of the Agricultural Research Council (C.R.M.) and the Science Research Council (A.P.K.) is gratefully acknowledged. We are also grateful to Technicon Instruments Co. for the loan of Auto-Analyzer equipment, to Dr D. S. Pepper, of the American National Red Cross, Washington, D.C. for the gift of serum glycoprotein, the donor of the seed viruses (Dr H. G. Pereira, WHO Influenza Centre, Mill Hill, England) and the Technical Staff of the Central Veterinary Laboratory, New Haw, Weybridge.

REFERENCES


(Received 15 March 1971)