Studies with Avian
Influenza A Viruses: Serological Relations of the Haemagglutinin
and Neuraminidase Antigens of Ten Virus Isolates

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SUMMARY

Ten strains of avian influenza A viruses, naturally pathogenic for birds and
grown in chick embryo allantois were compared by haemagglutination-inhibition
and neuraminidase-inhibition tests, using chicken antisera. The viruses segregated
into different cross-reaction groups in the two tests, emphasizing the need to include
an assessment of neuraminidase antigens in the taxonomy of influenza A viruses.
Four major groups of viruses were observed in the haemagglutination-inhibition
test but several very low titre cross-reactions were constantly found outside these
groups. Some of these reactions, not all of which were two-way, corresponded to
anti-neuraminidase cross-reactions. The results of neuraminidase-inhibition tests,
using a largely automated technique and a human serum fraction as substrate,
confirmed our previous results obtained with a smaller substrate. The virus neu-
raminidases could be separated into five separate antigen groups, without cross-
reactions between the groups.

INTRODUCTION

Influenza A viruses have been isolated from several species of domestic birds and from
some wild birds (Pereira, Tumova & Law, 1965; Pereira et al. 1966a; Pereira, Rinaldi
& Nardelli, 1966b). They possess a common internal ribonucleoprotein group antigen
which is also found in influenza A viruses isolated from human, equine, and porcine sources
tests with mammalian (Lief & Cohen, 1965) and human strains (Pereira, Tumova & Webster,
1967). These related antigens were shown to be the surface neuraminidase (Pereira et al.
1967; Webster & Pereira, 1968) rather than the haemagglutinin, and the results of Paniker
(1968) and Coleman et al. (1968) showed the importance of considering both the haemagg-
lutinin and neuraminidase antigens in characterizing new strains. Accordingly, an examination
of avian strains by haemagglutinin-inhibition (HI) and neuraminidase-inhibition (NI) tests
is necessary to establish their interrelationships and to provide a basis for comparison with
human and other strains. Such characterization is particularly important as new human
strains may arise from recombination between human and avian or animal strains (Webster
& Pereira, 1968). Some avian strains have been compared by strain specific complement-
fixation, HI and neutralization tests (Pereira et al. 1965, 1966a, b). Antigenic groupings were found which were similar in each test, but the authors compared only a few strains by NI (Webster & Pereira, 1968).

We have reported (Kendal & Madeley, 1969) an examination of ten strains of avian influenza viruses by NI using bovine sialo-lactose as substrate. It has been suggested (Fazekas de St Groth, 1963; Rafelson, Schneir & Wilson, 1963) that this material is too small to be a good substrate for NI tests. We have therefore repeated our experiments using a larger molecular weight substrate, Cohn fraction IV-4 of human serum. We report here the results of HI tests on these strains, together with NI tests using a new substrate.

**METHODS**

**Virus strains.** The viruses used, and their passage histories where known, are given in Table 1. Each seed virus from the source indicated was passed once or twice in the allantoic cavity of 10- to 11-day chick embryos in the presence of penicillin and streptomycin. The harvested allantoic fluid was stored in 0.5 ml. volumes in ampoules at -70° as stock virus. For each test, one ampoule was thawed and passed once in eggs at a 10^-3 dilution, each virus being handled in a separate room to avoid cross-contamination. Where necessary, virus was concentrated in a Spinco model L2 centrifuge using the type 19 rotor at 15,000 rev./min. for 90 min.

**Preparation of antisera.** Antisera were prepared in 4- to 5-week-old chickens (12-week-old for virus N) using groups of 20 to 30 birds for each virus. They were kept strictly isolated in units with separate ventilation systems at the Central Veterinary Laboratory, Weybridge. All injections were given intramuscularly but several schedules were used, as the viruses differed considerably in pathogenicity (Table 2). Schedule A was used with viruses lethal for the chicken, and, despite the pre-immunization, there was considerable mortality in these groups after the administration of live virus. With the viruses in Schedule B, a reinforcing dose was necessary to give adequate antibody titres. In all serological tests pooled antisera were used, each pool containing serum from at least four birds.

**Haemagglutination titration.** Haemagglutinin was titrated by the Takatsy Microtitre method using 0.025 ml. volumes and fowl erythrocytes at a final concentration of 0.5%. End points were read as the highest dilution showing complete agglutination. One haemagglutinin unit was defined as the amount of virus in 0.025 ml. which just caused complete haemagglutination.

**Haemagglutination-inhibition tests.** These were also done by the Takatsy method; 0.025 ml. of serum dilutions were mixed with eight haemagglutinin units in 0.025 ml. The mixtures were incubated under cover at 37° for 40 min. before the addition of 0.025 ml. 1% fowl cells. End points were read as the highest dilution showing complete inhibition of haemagglutination.

**Neuraminidase-inhibition tests.** Full details of the automated procedure used for these tests were published by Kendal & Madeley (1969). In the experiments reported here the substrate was human serum glyco-protein (Cohn Serum fraction IV-4). Untreated chicken serum was added to virus + substrate mixtures to give final serum dilutions of 1/50 to 1/1000. A graph was constructed of final extinction against dilutions of serum and the dilution of serum giving a 50% reduction of control virus activity was obtained by inspection. Normal serum controls were included.
### Table 1. Details of virus strains

<table>
<thead>
<tr>
<th>Virus</th>
<th>Common or other names</th>
<th>Seed obtained from</th>
<th>Passage history at receipt</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/BRESICA</td>
<td>Ascoli-brescia</td>
<td>Professor C. Hallauer, University of Berne, Berne, Switzerland</td>
<td>Isolated from chickens in Italy in 1902. Passed 118 times in eggs in Berne</td>
<td>Hallauer, 1947</td>
</tr>
<tr>
<td>A/DUTCH</td>
<td>Dutch, Classical fowl plague</td>
<td>Dr H. G. Pereira, WHO Influenza Centre, Mill Hill, England</td>
<td>Detailed history not known. Thought to have been isolated from chickens in Holland. (T. M. Doyle, personal communication)</td>
<td>Pereira et al., 1965</td>
</tr>
<tr>
<td>Virus N</td>
<td>None</td>
<td>Dr H. G. Pereira</td>
<td>Isolated from chickens in Germany in 1949. Passed 75 times in eggs</td>
<td>Dinter, 1949</td>
</tr>
<tr>
<td>A/ROSTOCK</td>
<td>Rostock</td>
<td>Professor W. Schäfer, Max-Planck-Institut für Virusforschung, Tübingen, W. Germany</td>
<td>Isolated from chickens in Rostock, East Germany, in 1934 by Professor Poppe. Passed in chickens until 1940, thereafter in eggs. Number of passes not known</td>
<td>Schäfer &amp; Schramm, 1950</td>
</tr>
<tr>
<td>A/CHICKEN/SCOTLAND/1959</td>
<td>Smith, Wilson</td>
<td>Dr H. G. Pereira</td>
<td>Isolated from chickens in Aberdeenshire, Scotland in 1959 by J. E. Wilson. Passed 9 times in eggs</td>
<td>Pereira et al., 1965</td>
</tr>
<tr>
<td>A/TURKEY/CANADA/1963</td>
<td>Wilmot/63</td>
<td>Professor G. Lang, University of Guelph, Ontario, Canada</td>
<td>Isolated from turkeys in Wilmot, Ontario, in 1963. Passed 6 times in eggs</td>
<td>Lang et al. 1965</td>
</tr>
<tr>
<td>Schedule A</td>
<td>Schedule B</td>
<td>Schedule C</td>
<td></td>
<td></td>
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<tr>
<td>------------</td>
<td>------------</td>
<td>------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>Action</td>
<td>Vaccine</td>
<td>Time</td>
<td>Action</td>
</tr>
<tr>
<td>0 weeks</td>
<td>1st vaccine dose</td>
<td>Allantoic fluid inactivated with 1/1000 formaldehyde. Dose 0.5 ml. IM (about 10^3 EID 50)</td>
<td>0 weeks</td>
<td>1st vaccine dose</td>
</tr>
<tr>
<td>2 weeks</td>
<td>2nd vaccine dose</td>
<td>10^3-3 EID 50 live virus as 1 ml. IM</td>
<td>2 weeks</td>
<td>Sample bleed</td>
</tr>
<tr>
<td>4 weeks</td>
<td>Serum obtained</td>
<td>—</td>
<td>4 weeks</td>
<td>2nd vaccine dose</td>
</tr>
<tr>
<td>6 weeks</td>
<td>Serum obtained</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Viruses used in each schedule:

A. A/Brescia, A/Dutch, A/Turkey/England/63
A/Rostock, A/Chicken/Scotland/59
A/Tern/South Africa/61

B. A/Turkey/Canada/63
A/Turkey/Canada/65
A/Turkey/England/66

C. Virus N
Table 3. *Haemagglutination-inhibition titres of avian influenza viruses and chicken immune sera*

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A/BREScia</td>
<td>512*</td>
<td>256</td>
<td>256</td>
<td>2048</td>
<td>4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A/DUTCH</td>
<td>128</td>
<td>1024</td>
<td>128</td>
<td>256</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
<td>A/TURKEY/ENGLAND/63</td>
<td>512</td>
<td>1024</td>
<td>2048</td>
<td>1024</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A/Rosstock</td>
<td>512</td>
<td>256</td>
<td>256</td>
<td>2048</td>
<td>4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A/CHICKEN/SCOTLAND/59</td>
<td>8</td>
<td>—</td>
<td>—</td>
<td>16</td>
<td>2048</td>
<td>5126</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>8</td>
</tr>
<tr>
<td>A/TURKEY/SOUTH AFRICA/61</td>
<td>—</td>
<td>—</td>
<td>4</td>
<td>—</td>
<td>5126</td>
<td>1024</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A/TURKEY/CANADA/63</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4</td>
<td>4</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>64</td>
<td>—</td>
</tr>
<tr>
<td>A/TURKEY/CANADA/65</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>16</td>
<td>8</td>
<td>256</td>
<td>256</td>
<td>128</td>
<td>256</td>
<td>—</td>
</tr>
<tr>
<td>A/TURKEY/ENGLAND/66</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>64</td>
<td>128</td>
<td>128</td>
<td>1024</td>
</tr>
</tbody>
</table>

* Highest dilution of serum giving complete inhibition of 8 haemagglutinin units of virus.

—, titre of 1/2. Normal sera gave no detectable inhibition in this test.
### Table 4. Neuraminidase inhibition titres of avian influenza viruses and chicken immune sera

<table>
<thead>
<tr>
<th>Virus antigen</th>
<th>Untreated immune sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/ROSTOCK</td>
<td>625</td>
</tr>
<tr>
<td>A/CHICKEN/SCOTLAND/59</td>
<td>225</td>
</tr>
<tr>
<td>A/BREScia</td>
<td>165</td>
</tr>
<tr>
<td>A/TURKEY/ENGLAND/63</td>
<td>—</td>
</tr>
<tr>
<td>A/TURN/SOUTH AFRICA/61</td>
<td>—</td>
</tr>
<tr>
<td>A/TURKEY/CANADA/63</td>
<td>—</td>
</tr>
<tr>
<td>A/TURKEY/CANADA/65</td>
<td>—</td>
</tr>
<tr>
<td>A/TURKEY/ENGLAND/66</td>
<td>—</td>
</tr>
<tr>
<td>A/DUTCH</td>
<td>—</td>
</tr>
<tr>
<td>Virus N</td>
<td>—</td>
</tr>
</tbody>
</table>

Figures are the dilution of the serum giving 50% inhibition of the control virus enzyme activity. Normal sera gave a titre of inhibition < 1/75. —, titre of < 1/75.
**RESULTS**

**Haemagglutination-inhibition**

Normal serum controls gave a titre of < 1/2 with all viruses. Preliminary experiments with immune sera showed that heating to 56°C did not affect their titres and, since some cross-reactions were of very low titre, it was not possible to treat the sera with periodate or receptor-destroying enzyme without eliminating these low titre reactions simply by dilution. However, high titre serum cross-reactions were not reduced more than twofold following treatment with periodate by the method of Robinson & Dowdle (1969), and, accordingly, all sera were used untreated. The viruses could be arranged into four groups by HI tests (Table 3). Within each group the titres of serum cross-reactions were > 1/64. These groups were: group 1: A/BRESCIA, A/DUTCH, A/ROSTOCK, and A/TURKEY/ENGLAND/63; group 2: A/TERN/SOUTH AFRICA/61 and A/CHICKEN/SCOTLAND/59; group 3: A/CANADA/63, A/CANADA/65, and A/TURKEY/ENGLAND/66; group 4: Virus N. Pereira *et al.* (1965; 1966a) used some of these viruses and, where comparisons are possible, their results agree with ours.

In addition to these major groupings, there were a number of cross-reactions with a titre of < 1/32. Some of these, e.g. A/TURKEY/ENGLAND/63 and A/TERN/SOUTH AFRICA/61, were one-way only. These reactions were reproducible, though always of low titre. No virus was inhibited by all sera and no serum inhibited all viruses. In contrast, an inhibitor-sensitive strain of human A2 influenza was inhibited by all sera in a similar test (C. R. Madeley, unpublished results).

**Neuraminidase-inhibition**

The ten viruses could be grouped on the basis of their neuraminidase antigens, namely group 1: A/BRESCIA, A/ROSTOCK, and A/CHICKEN/SCOTLAND/59; group 2: A/TURKEY/ENGLAND/63 and A/TERN/SOUTH AFRICA/61; group 3: A/TURKEY/CANADA/63; group 4: A/TURKEY/CANADA/65 and A/TURKEY/ENGLAND/66; group 5: A/DUTCH and Virus N. Outside these groupings no cross-reactions were detected. Normal sera gave 50% inhibition titres of up to 1/75 but even at 1/50 did not produce much more than 55% inhibition. In those tests (Table 4) shown as negative the sera gave no greater inhibition than that shown by normal sera. We found previously (Kendal & Madeley, 1969) that even low titre specific antibody inhibited up to 80%, suggesting that no specific cross-reactions were masked by the inhibitory activity of normal serum components. The present NI tests also confirmed completely our previous findings using the smaller sialo-lactose as enzyme substrate (Kendal & Madeley, 1969), and in our system the size of the enzyme substrate did not appear to affect the results of the test.

**DISCUSSION**

The particular combination of virus strains studied here has not been used by other workers, so that only a partial comparison may be made with their results. Where comparisons can be made, our results confirm those of Pereira *et al.* 1965, 1966a. They found that strains of DUTCH and TURKEY/ENGLAND/63 had a similar haemagglutinin, distinct from the TERN/SOUTH AFRICA/61 and CHICKEN/SCOTLAND/59 groups, and from the other Turkey strains. They did, however, find a low one-way cross-reaction between DUTCH and Virus N. Other low-titre cross-reactions that we have observed were not reported, either because they did not use all of the viruses concerned or, perhaps, because the titres were below their starting dilution of 1/10.
Low titre HI cross-reactions might be due to other factors than antibody specific to haemagglutinin. Since we used an entirely ‘chicken’ system the low reactions shown in Table 3 could not be due to any anti-species antibody. The effect of non-specific inhibitors is difficult to assess, since the standard procedures for serum treatment introduce a significant dilution factor. Heating did not involve dilution but made no difference to the titres. Fowl serum inhibitors of human influenza strains are not sensitive to heat, and in some cases the titre may be increased by heating to 56°C (Ananthanarayan & Paniker, 1960). Since the cross-reactions in Table 3 were consistently present, even with different serum pools, and as individual pooled sera also gave negative results with other viruses, we feel that they represent genuine inhibition due to antibodies.

Some of these cross-reactions might have resulted from the presence of anti-neuraminidase antibodies. Comparison of Tables 3 and 4 shows that cross-reactions between CHICKEN/SCOTLAND/59, A/ROSTOCK and A/BRESCIA, between TURKEY/ENGLAND/63 and TERN/SOUTH AFRICA/61, and between DUTCH and virus N, are explicable on the basis of anti-neuraminidase antibody interfering with haemagglutination. Such antibody may not necessarily inhibit haemagglutination (Seto & Rott, 1966; Kendal & Madeley, 1970), and not all minor cross-reactions can be accounted for in this way. In particular, the reactions between TURKEY/CANADA/63 virus and TERN/SOUTH AFRICA/61 serum and between TURKEY/CANADA/65 virus and CHICKEN/SCOTLAND/59 serum are not so explained.

Conversely, even a high titre of anti-haemagglutinin, e.g. TURKEY/ENGLAND/63 versus A/ROSTOCK and A/DUTCH versus A/BRESCIA, did not inhibit neuraminidase in the present tests. This has been noted already for some other virus strains (Seto & Rott, 1966; Webster & Laver, 1967), and these results suggest that such spatial separation may exist between haemagglutinin and neuraminidase on the virus surface that there is little or no steric hindrance of one by antibody attached to the other. It has been suggested (Compans, Dimmock & Meier-Ewert, 1969; Apostolov, Flewett & Kendal, 1970; Kendal & Madeley, 1970) that neuraminidase may be present in discrete enclaves on the surface of the virus particles rather than randomly distributed, and it is possible that different viruses vary in the detailed arrangement of haemagglutinin and neuraminidase on their surface. Consequently, the surface antigens of some viruses may be more susceptible to the steric hindrance effects of antibody than others, resulting in one-way cross-reactions. Similarly, HI antibody is less likely to inhibit neuraminidase, even with a large substrate, if the enzyme is located in an enclave. We did not observe any qualitative differences using a larger substrate, and all cross-reactions using the smaller substrate, sialo-lactose, have been confirmed using glycoprotein. No new cross-reactions were found.

Our experiments indicate that the relationships among the avian influenza viruses are complex. In particular, viruses previously believed to be the same on the basis of HI results have now been shown to possess different neuraminidases, e.g. A/DUTCH, A/ROSTOCK and A/TURKEY/ENGLAND/63. Similarly, A/TERN/SOUTH AFRICA/61 and A/CHICKEN/SCOTLAND/59 are also shown to be dissimilar. These results also confirm that the haemagglutinin and neuraminidase antigens in influenza viruses vary independently. The observations that similar neuraminidase antigens have been found in human and avian strains (Webster & Pereira, 1968; Schild, Pereira & Schettler, 1969) can be interpreted in two ways. Either there is an interchange, by recombination, of antigens between viruses from different species or the number of antigenic alternatives is limited. This latter has been postulated (Jensen & Francis, 1953), but not proven, for the haemagglutinin antigens, but now seems more likely to apply to neuraminidase antigens. At present there is insufficient evidence to support conclusively either hypothesis but the recurrence of an older type of neuraminidase in
Avian influenza viruses

A recent turkey isolate (Kendal, Madeley & Allan, unpublished results) may provide some support for a limitation of possible antigenic alternatives. Further information from fresh isolates will help to establish which of the hypotheses outlined above is correct. The results described here provide a basis for the characterization of new virus isolates.

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REFERENCES


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