J. gen. Virol. (1968), 3, 201–208
Printed in Great Britain

A Common Surface Antigen in Influenza Viruses from Human and Avian Sources

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(*Accepted* 20 *February* 1968)

SUMMARY

Serological cross-reactions were detected between four avian influenza viruses and a human A 2/57 virus and between an avian (TURKEY/CANADA/63) and an equine (EQUI 2/MIAMI/63) strain. The reactions were detected by strain-specific complement fixation tests and neuraminidase inhibition but not by haemagglutination-inhibition. Specific antiserum to the neuraminidase of an A 2/57 virus also reacted with the four avian viruses showing that it was the neuraminidase antigen on the avian and human A 2 virus that were antigenically closely related. The possibility that these viruses may have arisen by recombination between human and avian influenza viruses in nature is discussed.

INTRODUCTION

Influenza A viruses isolated from human, swine, horses and birds have been grouped together on the basis of antigenic similarities of the internal ribonucleoprotein antigen (S or G antigen), but until recently few if any relationships have been found between the external antigens of human and animal influenza viruses. Serological relationships have been demonstrated, however, between the V antigens of certain influenza viruses of avian and mammalian origins (Lief & Cohen, 1965; Tumova & Pereira, 1968). The antigenic similarity between the external antigens of a human and an avian influenza virus (Pereira, Tumova & Webster, 1967*b*) shows that such relationships may be more common than was previously thought. In the work reported here it was found that several influenza viruses from turkeys and ducks have an external antigen immunologically indistinguishable from one of the external antigens in human A 2 influenza virus. A similar relationship was found between an avian and an equine influenza virus. There are at least two virus-coded antigens on the surface of the influenza virion and the results show that it is the neuraminidase antigen which is common to the avian and human influenza viruses.

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METHODS

Viruses

The following strains of influenza virus were used in this study:

(1) EQUI 2/MIAMI/1/63,
(2) TURKEY/CANADA/63 (WILMOT strain),
(3) TURKEY/MASSACHUSETTS/3740/65,
(4) TURKEY/WISCONSIN/66,
(5) DUCK/ITALY/574/66,
(6) DUCK/ITALY/946/66,
(7) A 2/SINGAPORE/1/57,
(8) X-7 (F 1) (Kilbourne *et al.* 1967).

The origin of strains 1 to 7 have been described by Pereira *et al.* (1966) and Pereira, Rinaldi & Nardelli, (1967*a*). An inhibitor-insensitive line of A 2/SINGAPORE/1/57 was used in haemagglutination-inhibition tests. The viruses were grown in the allantois of 11-day-old chick embryos and concentrated by adsorption to and elution from chick erythrocytes. Infective allantoic fluids and virus concentrates were stored at −70°.

Isolation of neuraminidase from X-7 (F 1) virus

The neuraminidase of A 2/R $1/5^+$ virus was isolated from the recombinant influenza virus X-7 (F 1) (Kilbourne *et al.* 1967). The X-7 (F 1) virus was disrupted with sodium dodecyl sulphate and the proteins were separated by electrophoresis on cellulose acetate (Laver, 1964; Webster, Laver & Kilbourne, 1968). The enzyme band was eluted with saline, and the enzyme was concentrated and freed from the detergent by precipitation with 3 vol. cold (−20°) acetone and dissolved in physiological saline.

Antisera

Post-infection antisera were prepared as described previously (Pereira *et al.* 1966); anti-V guinea-pig sera were prepared according to the technique described by Lief & Henle (1959); hyperimmune rabbit antisera were prepared as described by Webster & Laver (1967). Antiserum to the neuraminidase of A 2/R $1/5^+$ was prepared in rabbits (Kilbourne *et al.* 1968). The neuraminidase isolated from X-7 (F 1) virus was emulsified in incomplete Freund's adjuvant and injected intramuscularly into adult rabbits, followed by a second injection of isolated neuraminidase in adjuvant 40 days later. Blood samples were taken from the marginal ear vein before vaccination and 10 days after the second injection. Sera were stored at −15°.

Serological methods

Haemagglutination–inhibition tests (*HI*) were done by the microtitrator technique of Takatsy (1955). Serum + virus mixtures were kept at room temperature for 1 hr before adding the erythrocyte suspension.

Complement-fixation tests (CF) were done in plastic trays, as described by Pereira, Pereira & Law (1964).

Neuraminidase assays. Virus samples for neuraminidase assay (0·05 ml.) were diluted with an equal volume of saline (0·05 ml.) and incubated at 37° for 30 min. with 0·1 ml. of buffered fetuin solution (1·25 mg. in 0·1 M-sodium phosphate, pH 5·9).

Liberated *N*-acetyl neuraminic acid was assayed by the method of Warren (1959), except that the colour was extracted into *n*-butanol containing 5 % (v/v) concentrated hydrochloric acid (Aminoff, 1961). One unit of enzyme is defined as that amount which will liberate sufficient *N*-acetyl neuraminic acid under standard conditions in 30 min. to give an extinction reading of 0·50 in a 1 cm. cell at a wavelength of 549 mμ. Fetuin was prepared by the method of Graham (1961).

Neuraminidase inhibition test (*NI*): Influenza viruses (containing 1 to 1·5 units of enzyme in 0·05 ml.) were incubated with 0·05 ml. volumes of serial dilutions of antisera at 37° for 30 min. when 0·1 ml. of buffered fetuin solution (1·25 mg. in 0·1 M-sodium phosphate, pH 5·9) was added and the mixture incubated at 37° for 30 min. Pre-immunization sera at equivalent dilutions were added to control samples of enzyme. This was necessary because the activity of this enzyme is often enhanced in the presence of normal serum (Ada, Lind & Laver, 1963; Fazekas de St Groth, 1963). The percentage inhibition of neuraminidase was plotted against the serum dilutions, and a linear relationship was usually obtained in the range of the test. The neuraminidase inhibition titre of a serum was expressed as the reciprocal of the dilution of the serum added to the reaction mixture inhibiting 50 % of the enzymic activity.

RESULTS

On the basis of the HI test (Table 1) the influenza viruses tested could be separated into equine, avian and human strains; antibody to the A 2 neuraminidase failed to react in HI tests. The turkey strains of influenza virus from North America (TURKEY/CANADA/63, TURKEY/MASSACHUSETTS/65, TURKEY/WISCONSIN/66) and the recently isolated duck strains from Italy (DUCK/ITALY/574/66, DUCK/ITALY/946/66) could be distinguished from one another on the basis of the HI test, but they are still directly or indirectly related to each other as has been shown previously (Pereira *et al.* 1966; Pereira *et al.* 1967*a*). High-titre postinfection antiserum to TURKEY/WISCONSIN/66 reacted weakly in HI tests w th A 2/SINGAPORE/1/57 but there was no reciprocal cross-reaction; antiserum to A 2/SINGAPORE/1/57 reacted significantly only with the homologous virus and anti-serum to the neuraminidase of a 1957 A 2 human influenza virus (R $1/5^+$) did not react in HI tests even with A 2/SINGAPORE/1/57. No crossing was observed by HI between EQUI 2/MIAMI/63 and the avian or human strains tested.

On the basis of CF and NI tests (Table 2, 3) the viruses tested could be separated into two groups: EQUI 2/MIAMI/63 and TURKEY/CANADA/63 formed one group while TURKEY/MASSACHUSETTS/65, TURKEY/WISCONSIN/66, DUCK/ITALY/574/66, DUCK/ITALY/946/66 and A 2/SINGAPORE/1/57 formed the second group.

Both the CF and NI tests (Table 2, 3) showed cross-reactions between the turkey and duck strains of influenza virus tested (TURKEY/MASSACHUSETTS/65, TURKEY/WISCONSIN/66, DUCK/ITALY/574/66 and DUCK/ITALY/946/66) and these viruses cross-reacted with A 2/SINGAPORE/1/57. Antiserum specific for the neuraminidase of a 1957 A 2 virus (RI/5^+) reacted with the above-mentioned turkey and duck strains and with the A 2/SINGAPORE/1/57 virus to similar titres both in the CF and NI tests. The low titres (1/10) found with specific anti-A 2 neuraminidase in the CF test with EQUI 2/MIAMI/63 and TURKEY/CANADA/63 viruses are probably not significant for they are close to the anticomplementary range of normal rabbit serum.

There was a remarkable degree of correlation between the CF and NI results

Table 1. *Haemagglutination-inhibition test*

	Antisera											
	EQUI 2/MIAMI/63		TURKEY/CANADA/63		TURKEY/MASSACHUSETTS/65		TURKEY/WISCONSIN/66		DUCK/ITALY/574/66	A 2/SINGAPORE/1/57		
	Ferret 43/63 PI†	Guinea pig 11/63 GP-V‡	Chicken 1/66 PI	Guinea pig 27/64 GP-V	Chicken 1/67 PI	Rabbit 5/67 R-H§	Turkey (1) PI	Guinea pig 11/66 GP-V	Guinea pig 39/67 GP-V	Ferret 25/65 PI	Rabbit 4/67 R-H	a-E/57* Rabbit 2
EQUI 2/MIAMI/63	640	<	<	<	<	<	<	<	<	<	<	<
TURKEY/CANADA/63	<	<	240	<	<	<	320	<	<	<	<	<
TURKEY/MASSACHUSETTS/65	<	<	60	<	320	480	240	<	15	<	<	<
TURKEY/WISCONSIN/66	<	<	<	<	<	<	> 1280	80	60	<	<	<
DUCK/ITALY/574/66	<	<	<	<	<	<	> 160	<	> 1280	<	20	<
A 2/SINGAPORE/1/57	<	<	<	<	<	<	10	<	<	640	480	<
DUCK/ITALY/946/66	<	<	<	<	<	<	160	<	> 1280	<	<	<

Figures represent the reciprocal of serum dilutions estimated to cause 50 % inhibition of haemagglutination. < = less than 1/10.

* Specific A 2/R1/5$^+$ antineuraminidase from X-7 (F1) virus (see Methods). † Postinfection. ‡ Guinea pig anti-V sera. § Rabbit hyperimmune.

Table 2. *Complement fixation*

Antigen	Antisera								
	EQUI 2/MIAMI/63	TURKEY/CANADA/63	TURKEY/MASSA-CHUSETTS/65		TURKEY/WISCONSIN/66	DUCK/ITALY/574/66	A 2/SINGAPORE/1/57		
	Guinea pig 11/63 GP-V	Guinea pig 27/64 GP-V	Rabbit 5/67 R-H	Guinea pig 7/66 GP-V	Guinea pig 11/66 GP-V	Guinea pig 39/67 GP-V	Rabbit 4-67 R-H	Guinea pig 3/66 GP-V	a-E/57* Rabbit 2
EQUI 2/MIAMI/63	80	120	NT	<	<	< 40	NT	<	10
TURKEY/CANADA/63	<	160	NT	120	<	< 40	NT	<	10
TURKEY/MASSA-CHUSETTS/65	<	<	NT	960	15	240	NT	160	640
TURKEY/WISCONSIN/66	<	<	480	480	160	240	NT	480	320
DUCK/ITALY/574/66	<	<	NT	320	<	10240	NT	160	480
A 2/SINGAPORE/1/57	<	<	NT	160	<	640	960	> 1280	480
DUCK/ITALY/946/66	<	<	NT	240	<	240	NT	204	480

Figures represent the reciprocal of the highest serum dilution giving 50 % fixation with any of the antigen dilutions used.

* Specific A 2/RI/5⁺ antineuraminidase from X-7 (F I) virus (see Methods). NT = not tested; < = less than 1/10.

Table 3. *Neuraminidase inhibition tests*

Virus enzyme	Antisera						
	EQUI 2/MIAMI/63 Guinea pig 12/63	TURKEY/CANADA/63 Guinea pig 27/64	TURKEY/MASSA-CHUSETTS/65 Rabbit 5/67	TURKEY/WISCONSIN/66 Guinea pig 11/66	DUCK/ITALY/574/66 Guinea pig 39/67	A 2/SINGA-PORE/1/57 Rabbit 4/67	a-E/57* Rabbit 2
EQUI 2/MIAMI/63	36	72	<	<	<	<	<
TURKEY/CANADA/63	16	270	12	<	<	<	<
TURKEY/MASSACHUSETTS/65	<	<	820	10	30	680	800
TURKEY/WISCONSIN/66	<	<	270	12	30	100	400
DUCK/ITALY/574/66	<	<	250	10	200	150	600
A 2/SINGAPORE/1/57	<	<	800	52	160	950	1200
DUCK/ITALY/946/66	<	<	400	10	350	160	1000

Values are initial dilutions (× 4 for final) and show the dilution of serum causing 50 % inhibition of enzyme activity.

* Specific A 2/RI/5⁺ antineuraminidase from X-7 (F 1) virus (see Methods). < = less than 1/10.

(Tables 2, 3); the only place where the two tests did not correlate was in the test between TURKEY/CANADA/63 virus and antiserum to EQUI/MIAMI/63 (guinea pig number 12/63). NI tests showed reciprocal reaction between these viruses and antisera while CF tests showed a one-way cross. This may have been due to the fact that it was necessary to use different antisera in the CF and NI tests.

Comparison of the results from the three serological tests showed that HI tests did not detect significant cross-reactions between EQUI 2/MIAMI/63 and TURKEY/CANADA/63 nor between the avian strains and the human A 2/SINGAPORE/1/57. It is also interesting to note that some guinea-pig anti-V antisera (EQUI 2/MIAMI/guinea pig 11/63 and TURKEY/CANADA/63/guinea pig 27/64) failed to inhibit even the homologous virus in HI tests although they had reasonable titres in CF or NI tests. After immunization with influenza-virus envelope antigens obtained by ether treatment and purification by adsorption and elution (Lief & Henle, 1959) most guinea pigs produce antibodies against both V antigens of influenza virus (the haemagglutinin and the neuraminidase) but some guinea pigs evidently responded to only one antigen, in this case the neuraminidase.

DISCUSSION

The recognition of four avian viruses from different parts of the world (two from U.S.A. and two from Italy) possessing a neuraminidase antigen serologically related to the neuraminidase of A 2/SINGAPORE/1/57 virus confirms the earlier observation of Pereira *et al.* (1967*b*). The independent isolation of four avian viruses also supports the belief that these viruses are not mixtures, and did not arise in the laboratory by accidental mixing and recombination.

Recombinant influenza viruses with similar properties to the avian viruses described above can be produced in the laboratory by mixedly infecting cells with different influenza virus strains and selecting the resulting progeny. Recombinants between different human influenza strains (Kilbourne *et al.* 1967), and between animal and human strains (Tumova & Pereira, 1965; Kilbourne, personal communication), have been isolated in the laboratory. The recombinant influenza viruses that are antigenic hybrids usually contain the haemagglutinin from one parent and the neuraminidase from the other (Laver & Kilbourne, 1966; Kilbourne *et al.* 1967; Easterday & Pereira, unpublished data).

In general, antibodies to the neuraminidase of influenza viruses react in CF and NI tests (Webster *et al.* 1968) but do not cause HI. Antibodies to the haemagglutinin react in CF and in HI tests but can also cause low levels of NI at very high concentrations of antibody, presumably by steric inhibition. This may also explain the small cross-reactions revealed between A 2/SINGAPORE/1/57 and TURKEY/MASSACHUSETTS/65 by HI tests done with hyperimmune sera (Pereira *et al.* 1967*b*). There are exceptions to these general findings; a recombinant influenza virus (X-15) possessing equine influenza haemagglutinin and human A 2 neuraminidase was inhibited in HI tests by antineuraminidase antibodies (Kilbourne, personal communication). In most instances CF tests should detect reactions between both antigens on the surface of influenza viruses while NI tests should detect the neuraminidase and HI tests the haemagglutinin. Consideration should also be given to the possible role that the host component incorporated in the viral envelope may play in cross-reactions observed between influenza viruses. This component can be revealed by HI (Harboe, Borthne &

Berg, 1961) and presumably also by complement fixation. Although the present findings can be explained without postulating that this additional factor is involved, its role should be kept in mind in similar studies.

Several hypotheses can be formulated to account for the occurrence on the avian viruses of a neuraminidase antigenically related to the human A 2/57 enzyme. One is that this human subtype may have been derived from an avian influenza virus by a process of host range mutation. An alternative hypothesis is that the antigenic determinants on the neuraminidases of the human and avian influenza viruses may have arisen by a series of independent mutations and the antigenic similarity is a chance occurrence. Finally, it may have resulted from recombination occurring in nature between an avian virus and a human virus possessing the A 2/57 type of neuraminidase. In view of the relative ease with which antigenic hybrids of influenza viruses can be made 'to order' in the laboratory (Kilbourne, personal communication) it is tempting to suggest that the avian viruses with the A 2/57 type neuraminidase arose by the last mechanism and one can speculate on the potentially large reservoir of animal influenza viruses with which human strains could recombine to give rise to a new pandemic strain.

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(*Received* 2 *February* 1968)