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Relationship of Envelope Antigens of Animal Influenza Viruses to Human A2 Influenza Strains Isolated in the Years 1957–68*

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This study demonstrates relationships in envelope antigens of 4 human influenza A2 strains isolated during the period 1957–68 (including A2/Hong Kong/68), 2 strains of A/Equi-2/63 and 7 avian influenza viruses isolated in Europe, North America, and the Ukraine in the years 1960–67.

Antigenic relationships among the strains were determined on the basis of haemagglutination-inhibition, virus-neutralization, strain-specific complement-fixation, and neuraminidase-inhibition tests.

North American avian influenza strains, Turkey/California/64, Turkey/Massachusetts/65, Turkey/Wisconsin/66, Turkey/Ontario/6828/67, and the Italian strain Duck/Italy/574/66 are antigenically related to human A2 influenza viruses by haemagglutinin and/or neuraminidase. None of these viruses is antigenically related to the A/Equi-2/63 strains, Duck/Ukraine/2/60, Duck/Ukraine/1/63 or A2/Hong Kong/68. However, A2/Hong Kong/68 has neuraminidase similar to other A2 strains from previous years.

A definite relationship was shown between the haemagglutinin of A/Equi-2/63, A2/Hong Kong/68, Duck/Ukraine/2/60 and Duck/Ukraine/1/63 strains by the use of hyperimmune sera in 3 different serological tests. Related neuraminidase was demonstrated only between A/Equi-2/63 and both duck strains from the Ukraine.

The significance of these findings and their interpretation with respect to the ecology of influenza viruses are discussed.

The origin and evolution of antigenic changes in influenza virus type A remain poorly understood and several hypotheses proposed do not satisfactorily explain the appearance of variant or novel subtypes. The hypothesis of a common origin of animal and human type A influenza viruses has received considerable support in recent years with the recovery of a number of strains from different animal species related to the A2 subtype human influenza viruses by one or both envelope antigens, haemagglutinin and neuraminidase.

Pereira, Tumova & Webster (1967) and Tumova & Pereira (1968) demonstrated the similarity of neuraminidase in the strains Turkey/Massachusetts/65 and A2/Singapore/57. Later, Webster & Pereira (1968) described 4 additional strains originally isolated from turkeys and ducks which shared neuraminidase antigens with A2 human influenza virus.

This paper describes (1) antigenic relationships of surface antigens between some avian influenza strains isolated in Italy and North America and A2 human influenza strains isolated prior to the year 1968, and (2) antigenic relationships of haemagglutinin and/or neuraminidase of the A2/Hong Kong/68 variant to 7 avian, 2 equine, and 3 human A2 influenza strains.

MATERIALS AND METHODS

Viruses

The following strains were employed in this study (abbreviations used in the tables are given in parentheses).

Human influenza viruses. A2/Singapore/1/57 (A2/Singap./57), A2/Bratislava/65 (A2/Bratisl./65), A2/Olomouc/1/68, and A2/Hong Kong/1/68.

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All strains were isolated from outbreaks of influenza in the Far East and in Czechoslovakia (Coleman et al., 1968).

Equine influenza viruses. A/Equi-2/Detroit/63 (A/Equi-2/63), A/Equi-2/Miami/63.
Because of the previously described similarity of A/Equi-2 strains, only one strain (A/Equi-2/Detroit/63) was used in most tests (Waddell et al., 1963; Dowdle et al., 1963).

Avian influenza viruses. Turkey/California/64 (Ty/Calif./64), Turkey/Massachusetts/65 (Ty/Mass./65), Turkey/Wisconsin/66 (Ty/Wis./66), Turkey/Ontario/6828/67 (Ty/Ont./6828/67), Duck/Italy/574/66 (Dk/It./574/66), Duck/Ukraine/2/60 (Dk/Ukr./2/60), Duck/Ukraine/1/63 (Dk/Ukr./1/63).
Strain history and characteristics have been described by Pereira, Tumova & Law (1965), Pereira, Lang et al. (1966), Pereira, Rinaldi & Nardelli (1967) and Easterday & Tumova (1970). An additional 16 avian influenza strains and corresponding antisera were used for screening of antigenic relationships. Negative results excluded them from further study: Fowl plague, Turkey/England/63, Virus N, Duck/Canada/53, Duck/England/56, Duck/Czechoslovakia/56, Duck/England/62, Turkey/Canada/63, Chicken/Scotland/59, Turkey/Italy/741/65, Pheasant/Italy/647/66, Quail/Italy/1117/65, Quail/Italy/544/66, Turkey/Alberta/6962/66, Turkey/Ontario/6118/67, Turkey/England/66, and equine influenza virus Equi-1/Praha/1/56.

All strains were propagated in embryonated eggs by allantoic inoculation. Allantoic fluid was used as antigen for the haemagglutination-inhibition test; antigens for the complement-fixation test were prepared by adsorption to and elution from chicken red blood cells. Neuraminidase (Nase) for the neuraminidase-inhibition test was concentrated from allantoic fluid by high-speed centrifugation (4 hours at 5.5×10^4 *g*) and the sedimented pellet was resuspended in saline to 1/10 of the original volume, and afterwards sonicated.

Antisera

Hyperimmune rat sera were prepared in rats as described by Tumova et al. (1963). Strain-specific V antisera were prepared in guinea-pigs by the technique described by Lief & Henle (1959).

Serological methods

Haemagglutination-inhibition (HI) tests were performed in plastic trays. Antisera were treated with receptor-destroying enzyme (RDE) (WHO Expert Committee on Respiratory Virus Diseases, 1959). The serum–virus mixtures were incubated at room temperature for 1 hour before addition of chick erythrocytes.

Complement-fixation (CF) tests were performed in plastic trays as described by Pereira et al. (1964).

Neuraminidase-inhibition (NI) tests were performed according to the method described by Webster & Pereira (1968).

Virus-neutralization (VN) tests were performed in monkey (*Cercopithecus aethiops*) kidney cells seeded with 80 000 cells per 1 ml of medium, and incubated in a stationary position for 48–72 hours. Eple's and Eagle's media, in a 1:1 ratio and supplemented with 10% calf serum, were used as the nutrient medium. Serum was serially diluted in maintenance medium (nutrient medium without the calf serum) and mixed with 10–100 haemadsorption units of virus. After 48 hours' incubation, the reaction was read by means of haemadsorption with guinea-pig erythrocytes.

Results of the serological tests are expressed as reciprocals of serum dilution at 50% end-point.

RESULTS

Antigenic relationships between the North American strains isolated from turkeys, the Italian strain Duck/Italy/574/66 and 2 strains of A2 human influenza were determined by means of HI, VN, or NI tests with hyperimmune rat sera and by strain-specific CF tests with guinea-pig sera. The results are shown in Tables 1 and 2. Inhibition of haemagglutination was demonstrated between A2 influenza strains and the sera against Turkey/California/64, Turkey/Wisconsin/66, and Duck/Italy/574/66. Reciprocal inhibition of haemagglutination and neutralization was demonstrated with the human A2 strain, Turkey/Massachusetts/65, and Turkey/Ontario/6828/67. There was no neutralization of A2 viruses with the Turkey/California/64 and Duck/Italy/574/66 antisera and only one-way neutralization by the Turkey/Wisconsin/66 antiserum. Results of NI tests suggest that all strains under study are related by neuraminidase except Turkey/Ontario/6828/67.

The neuraminidase of this strain is very little related to that of other avian strains tested or of A2/Singapore/57 as shown by CF and NI tests. A one-way reaction could be demonstrated only with Turkey/Ontario/6828/67 serum, while the neuraminidase of Turkey/Ontario/6828/67 was not inhibited by any hyperimmune sera to other strains.

TABLE 1

RELATIONSHIPS OF AVIAN INFLUENZA VIRUSES TO HUMAN A2 STRAINS, SHOWN BY RESULTS OF HAEMAGGLUTINATION-INHIBITION AND VIRUS NEUTRALIZATION TESTS [a]

Rat sera	Antigens													
	A2/Singap./57		A2/Bratisl./65		Ty/Calif./64		Ty/Mass./65		Ty/Wis./66		Dk/It./574/66		Ty/Ont./6828/67	
	HI	VN	HI	VN	HI	VN	HI	VN	HI	VN	HI	VN	HI	VN
A2/Singap./57	1 280	96	160	16			160						640	256
A2/Bratisl./65	10	32	1 280	192			20						40	24
Ty/Calif./64	10		20		1 280	256	640	384						
Ty/Mass./65	80	96	NT		320	256	640	1 024						
Ty/Wis./66	40	16	80	NT			160	32	640	768				
Dk/It./574/66	10		40	NT				8	10	16	1 280	768		
Ty/Ont./6828/67	640	256	40	NT		12							1 280	384

[a] Blank spaces denote negative results; NT = not tested.

TABLE 2

RELATIONSHIPS OF AVIAN INFLUENZA VIRUSES TO HUMAN A2 STRAINS, SHOWN BY RESULTS OF STRAIN-SPECIFIC COMPLEMENT-FIXATION AND NEURAMINIDASE-INHIBITION TESTS [a]

Sera	Antigens											
	A2/Singap./57		Ty/Calif./64		Ty/Mass./65		Ty/Wis./66		Dk/It./574/66		Ty/Ont./6828/67	
	CF	NI	CF	NI	CF	NI	CF	NI	CF	NI	CF	NI
A2/Singap./57	320	224		NT	40	316	30	125	30	178	40	
Ty/Calif./64		NT	320	NT	160	NT	15	NT		NT		NT
Ty/Mass./65	15	355	30	NT	120	400	15	316	15	250		
Ty/Wis./66	30	400	15	NT	40	500	120	1 000	30	1 000		
Dk/It./574/66	10	500		NT	80	630	80	560	320	590		
Ty/Ont./6828/67	40	40		NT	5	15		11		11	320	100
Control sera [b]	1 000			NT	>1 000		>1 000		>1 000		>10	

[a] Blank spaces denote negative results; NT = not tested; > = no end-point in the highest dilution tested.

[b] Guinea-pig anti-soluble A serum was used in CF tests with negative results; rabbit anti-A2/Singap./57 neuraminidase serum was used in NI tests.

However, the haemagglutinins of the Turkey/Ontario/6828/67 and A2/Singapore/57 viruses are closely related if not identical. Both strains differ also by haemagglutinin from the virus A2/Bratislava/65 to the same extent.

None of these strains tested by HI tests reacted with equine influenza virus type 1 and 2, A2/Hong Kong/1/68, or Duck/Ukraine/1/63.

The A2/Hong Kong/1/68 strain differs by haemagglutinin from A2 strains isolated in the years 1957–65 (Tables 3 and 4). Cross-reaction was shown only with the virus isolated from the influenza outbreak in Czechoslovakia in February–March 1968. All A2 strains share similar neuraminidase as demonstrated by NI tests (Table 3). However, there was no inhibition of haemagglutination by the North

TABLE 3
ANTIGENIC RELATIONSHIPS OF THE VIRUS A2/Hong Kong/1/68 SHOWN BY RESULTS OF HAEMAGGLUTINATION-INHIBITION AND NEURAMINIDASE-INHIBITION TESTS WITH A2/Hong Kong ANTIGEN [a]

Rat sera	HI	NI
A2/Hong Kong/68	1 280	260
A2/Singap./57	0	65
A2/Olomouc/68	10	70
Ty/Calif./64	0	0
Ty/Mass./65	0	0
Ty/Wis./66	0	10 [b]
Dk/It./574/66	0	10 [b]
Ty/Ont./6828/67	0	0
Rat control serum	0	0

[a] 0 = no inhibition in the 1 : 10 serum dilution.
[b] 41% inhibition in the 1 : 10 serum dilution.

American and Italian avian influenza viruses previously shown to be related to A2/Singapore/57 and A2/Bratislava/65. Only the strains Turkey/Wisconsin/66 and Duck/Italy/574/66 gave minimal inhibition of A2/Hong Kong/1/68 neuraminidase in the serum dilution 1:10. This weak reaction could hardly be considered a true relationship. Immune sera from other animal species, as shown by Webster & Pereira (1968), may give more definitive results.

An additional 17 animal influenza viruses (as listed under Materials and Methods above) and their corresponding antisera did not show any antigenic relationship to A2/Hong Kong/1/68 in HI tests.

Definite relationships in surface antigens were demonstrated by HI, VN, CF, and NI tests between the strains A2/Hong Kong/1/68, Duck/Ukraine/2/60 and Duck/Ukraine/1/63, and 2 strains of A/Equi-2 influenza virus (Tables 4, 5, 6). This relationship is due to the haemagglutinin that is shared by these viruses and is demonstrated in rat antisera inhibiting the haemagglutination and neutralizing A2/Hong Kong/1/68 virus. Similar results were obtained by HI and VN tests employing ferret Duck/Ukraine/2/60 and Duck/1/Ukraine/1/63 post-infectious sera.

It is clear that the neuraminidase of the A2/Hong Kong/1/68 virus is not related to the common neuraminidase of Duck/Ukraine/2/60, Duck/Ukraine/1/63 and the A/Equi-2 viruses (Table 6).

COMMENTS

Until recently most of the information pointing to a possible relationship between human and animal influenza was based on serological findings in human sera suggesting that animal viruses may have been responsible for human epidemics in the past. Direct antigenic relationship between human and animal infectious viruses was demonstrated between human

TABLE 4
ANTIGENIC RELATIONSHIPS OF THE VIRUS A2/Hong Kong/1/68, SHOWN BY RESULTS OF HAEMAGGLUTINATION-INHIBITION TESTS

Rat sera	Antigens							
	A2/Singap./57	A2/Bratisl./65	A2/Olomouc/1/63	A2/Hong Kong/1/68	Dk/Ukr./2/60	Dk/Ukr./1 63	A/Equi-2/Detroit/63	A/Equi-2/Miami/63
A2/Singap./57	1 280	160	60					
A2/Bratisl./65	10	320	160					
A2/Olomouc/1/68	10	160	320	10				
A2/Hong Kong/1/68			80	1 280				
Dk/Ukr./2/60				160	40	40	20	80
Dk/Ukr./1/63				640	80	640	120	80
A/Equi-2/Detroit/63				760	<10	<10	640	1 280
A/Equi-2/Miami/63				760	<10	<10	1 280	1 280

TABLE 5

ANTIGENIC RELATIONSHIP OF THE VIRUS A2/Hong Kong/1/68, SHOWN BY RESULTS OF VIRUS-NEUTRALIZATION TESTS IN MONKEY KIDNEY TISSUE CULTURES [a]

Sera	Antigens			
	A2/Hong Kong/1/68	Dk/Ukr./2/60	Dk/Ukr./1/63	A/Equi-2/63
A2/Hong Kong/1/68	960	30	10	40
Dk/Ukr./2/60	240	120	60	20
Dk/Ukr./1/63	>320	120	>320	60
A/Equi-2/63	>160	>40	10	>320
Control serum	0	0	0	0

[a] 0 = no neutralization in the dilution 1 : 10; > = no end-point.

TABLE 6

ANTIGENIC RELATIONSHIP OF THE VIRUS A2/Hong Kong/1/68, SHOWN BY RESULTS OF STRAIN-SPECIFIC COMPLEMENT-FIXATION AND NEURAMINIDASE-INHIBITION TESTS [a]

Sera	Antigens								
	A2/Hong Kong/1/68		Dk/Ukr./2/60		Dk/Ukr./1/63		A/Equi-2/63		Sol-A [b]
	CF	NI	CF	NI	CF	NI	CF	NI	CF
A2/Hong Kong/1/68	NT	260	NT	0	NT	0	NT	0	0
Dk/Ukr./2/60	70	0	160	400	20	310	20	340	0
Dk/Ukr./1/63	10	0	80	200	40	230	5	180	0
A/Equi-2/63	10	0	80	200	5	310	80	400	0
Control sera	0	0	0	0	0	0	0	0	160

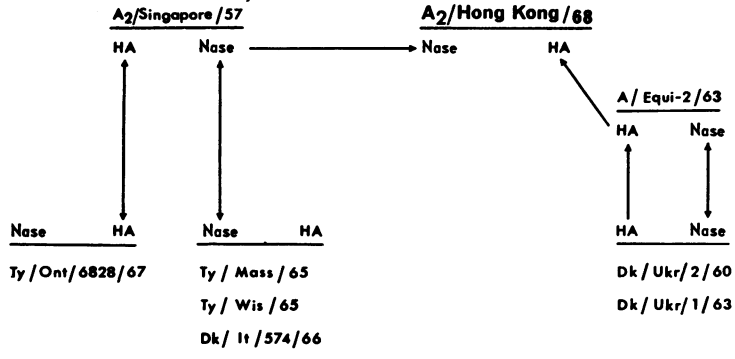
[a] 0 = negative results in the initial serum dilution 1 : 10; NT = not tested.
[b] Guinea-pig immune serum anti-soluble antigen.

A subtype and swine influenza strains and recently, between human A subtype, avian, and equine influenza viruses (Pereira, Tumova & Webster, 1967; Webster & Pereira, 1968; Kasel et al., 1969). Schild et al. (1969) described antigenic relationships between A0 and A1 subtypes of human influenza viruses and the strain A/Duck/Germany/1868/68. Antigenic similarity concerns mostly one of the surface antigens, neuraminidase, that is shared by human and animal influenza viruses. In this respect, our findings of common neuraminidase between 2 North American, Italian, and the A2/Singapore/57 influenza strains confirm previous observations of the other authors. There are some differences between our HI results and those of Webster & Pereira (1968), who used post-infectious sera in all tests. According to these authors, however, possible reactions between viruses in both HI and NI tests indicated common neuraminidase if hyperimmune sera with a high concentration of antibodies were employed.

A reciprocal relationship of common or similar haemagglutinin, such as between Turkey/Ontario/

6828/67 and A2/Singapore/57, is rather exceptional but significant with respect to laboratory diagnosis: both viruses could be considered either human or animal if tested only by HI. The similarity is expressed also in the same degree of relationship to the virus A2/Bratislava/65. This indicates the necessity of using both HI and NI tests in antigenic analysis studies. A search for a specific host component in the virus envelope (Harboe et al., 1966) might enable us to differentiate between the viruses of animal and human origin which are closely related by the surface antigens.

The relationship of A2/Hong Kong/1/68 virus to animal and human A2 strains is schematically expressed below (arrows indicate inhibition by specific antibody):



Similar haemagglutinin has been demonstrated in the Duck/Ukraine/2/60 and Duck/Ukraine/1/63 viruses and the A/Equi-2/63 virus (Kasel et al., 1969). Although the A2/Hong Kong/1/68 virus has a neuraminidase similar to A2/Singapore/57, there was no relationship between this strain and the Turkey/Massachusetts/65, Turkey/Wisconsin/66, and Duck/Italy/574/66 viruses.

The sharing of envelope antigen by strains from different hosts poses a question in relation to their origin, ecology, and evolution. With our present knowledge it is possible to postulate a common origin for all members of the A type of influenza viruses. However, it is still difficult to interpret the changes in the viral envelope antigen by either hypothesis proposed. The difference in relationship such as that between the neuraminidase of North American avian strains, A2/Singapore/57 and A2/Hong Kong/68 strains might suggest that influenza viruses undergo extensive variation concerning not only haemagglutinin but also neuraminidase and that evolution of human and animal viruses may be independent.

On the other hand, there is evidence that virus variants produced in the laboratory by means of recombination (Tumova & Pereira, 1965, Kilbourne et al., 1967; Easterday et al., 1969) contain haemagglutinin of animal and neuraminidase of human influenza viruses. We can speculate on a potentially large reservoir of animal viruses in nature—including the wild populations—with which human viruses could recombine either in an animal or human host. In the light of this speculation, A2/Hong Kong/68 virus could be considered a recombinant of either A/Equi-2/63 or Duck/Ukraine viruses and A2 human influenza.

Any of the present hypotheses may be shown in the future to be incorrect because of our present lack of knowledge about the distribution of animal viruses and also because of our lack of information about the antigenic structure of the influenza virus envelope.

REFERENCES

Coleman, M. T., Dowdle, W. R., Pereira, H. G., Schild, G. C. & Chang, W. K. (1968) *Lancet*, **2**, 1384-1387

Dowdle, W. R., Yarbrough, W. B. & Robinson, R. Q. (1963) *Publ. Hlth Rep. (Wash.)*, **79**, 398

Easterday, B. C., Laver, W. G., Pereira, H. G. & Schild, G. C. (1969) *J. gen. Virol.*, **5**, 83-91

Easterday, B. C. & Tumova, B. (1970) *Avian influenza.* In: Hofstad, M. J., ed., *Diseases of poultry*, Ames, Iowa State University Press (in press)

Harboe, A. R., Schoyen, R & Bye-Hanson, A. (1966) *Acta. path. microbiol. scand.*, **67**, 573-578

Kasel, J. A., Fulk, R. V. & Couch, R. B. (1969) *J. Immunol.*, **102**, 531-532

Kilbourne, E. D., Lief, F. S., Schulman, J. L., Jahiel, R. I. & Laver, W. G. (1967) *Prospect. Virol.*, **6**, 87

Lief, F. S. & Henle, W. (1959) *Bull. Wld Hlth Org.*, **20**, 411-420

Pereira, H. G., Lang, G., Olesiuk, O. M., Snoeyenbos, G. H., Roberts, D. H. & Easterday, B. C. (1966) *Bull. Wld Hlth Org.*, **35**, 799-802

Pereira, H. G., Pereira, M. S. & Law, V. G. (1964) *Bull. Wld Hlth Org.*, **31**, 129-132

Pereira, H. G., Rinaldi, A. & Nardelli, L. (1967) *Bull. Wld Hlth Org.*, **37**, 553-558

Pereira, H. G., Tumova, B. & Law, V. G. (1965) *Bull. Wld Hlth Org.*, **32**, 855-860

Pereira, H. G., Tumova, B. & Webster, R. G. (1967) *Nature (Lond.)*, **215**, 982-983

Schild, G. C., Pereira, H. G. & Schettler, C. H. (1969) *Nature (Lond.)*, **222**, 1299-1301

Tumova, B., Fedova, D., Boskova, D., Volenikova, Prochazkova, V. & Ludvik, J. (1963) *Acta. virol.*, **7**, 156-175

Tumova, B. & Pereira, H. G. (1965) *Virology*, **27**, 253

Tumova, B. & Pereira, H. G. (1968) *Bull. Wld Hlth Org.*, **38**, 415-420

Waddell, G. H., Teigland, M. B. & Sigel, M. M. (1963) *J. Amer. vet. med. Ass.*, **143**, 583

Webster, R. G. & Pereira, H. G. (1968) *J. gen. Virol.*, **3**, 201-208

WHO Expert Committee on Respiratory Virus Diseases (1959) *Wld Hlth Org. techn. Rep. Ser.*, **170**, 59