

## **Antigenic analysis of prototype influenza A (H3N2) strains by the antiserum absorption method**

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### SUMMARY

Prototype strains of the influenza A (H3N2) virus can be arranged on a gradient showing the degree of the antigenic drift which the haemagglutinins of the strains have undergone. The demonstration of fine antigenic differences is based on an antiserum absorption test which allows a detailed antigenic analysis of strains. The gradient provides information on variation in strains occurring in different geographical areas and its use may be helpful in differentiating between introduced strains and locally developing variants.

### INTRODUCTION

The differences between haemagglutinins of influenza virus strains are usually investigated by the cross haemagglutination-inhibition (HI) test. In general, the more time that has elapsed between the isolation of two strains of the same subtype the more pronounced antigenic difference is demonstrable between them, suggesting that the antigenic drift of the influenza virus is, in general, progressive. However, owing to different degrees of immunity of the populations in which different strains circulate, it may occur that strains isolated at the same time in different geographical regions are at different stages of progression or even a later strain may be at a less advanced stage. The cross HI test provides little information in this respect, especially late in the interpandemic period. Since, supposedly, the furthest progressed strains are of greater importance as regards future epidemics, the investigation of influenza virus strains from this point of view needs more sensitive tests. Such a test may be used in selecting the best strains for vaccine production.

In the present study several prototype strains of influenza A (H3N2) virus were investigated by an antiserum absorption test to determine their proper place on the gradient of antigenic drift. The method in general was described long ago (Takátsy & Fürész, 1954; Takátsy & Fürész, 1957; Takátsy & Barb, 1958) and has been somewhat modified for the present purpose.

## MATERIALS AND METHODS

*Influenza A (H3N2) virus strains*

International prototype strains Hong Kong/1/68, England/878/69, Hong Kong/107/71 and England/42/72 and strains Hungary/1/69, Hungary/20/70, Hungary/1/71, Hungary/92/71 isolated by us.

*Preparation of antisera*

Roosters were immunized intravenously with a purified virus suspension containing 3000 haemagglutination (HA) units per ml. Three 1 ml doses were administered at weekly intervals. Six to 8 weeks later a 1 ml. booster dose was given. The birds were exsanguinated 2 weeks after the last injection. The blood, taken with sodium citrate, was centrifuged and the plasma was inactivated at 56° C. for 30 min., then re-centrifuged. The supernatant plasma, with 0.05% sodium azide as preservative, was used as antiserum.

*Purified concentrated virus*

Allantoic fluid containing virus was mixed with a 2% suspension of formalized chicken erythrocytes. The mixture was kept at 4° C. for 30 min. while shaken at intervals. Finally, the virus was eluted from the sedimented erythrocytes in 5% NaCl at 37–40° C. The supernatant was dialysed against distilled water for 24–36 hr. By the end of this period the NaCl concentration in the inner fluid had decreased to between 0.2 and 0.3% and, consequently, the virus formed a precipitate, which was then sedimented by centrifugation at 3000 rev./min. The sediment was resuspended in phosphate-buffered saline (PBS) to obtain a virus suspension with a titre of approximately 10<sup>5</sup> HA units/ml. After centrifuging at 3000 rev./min. the supernatant of this suspension was used as purified concentrated virus (stock suspension).

*HA and HI tests*

Were carried out in the Microtitrator\* (Takátsy, 1955) apparatus.

*Antiserum absorption test*

Serial twofold virus dilutions were prepared from each stock suspension in two rows, using the 0.05 ml. Microtitrator loops. In the first well of the second row the dilution was 1/3 (one loopful in 0.1 ml diluent); in all the other wells the volume of the diluent was 0.05 ml. After the dilution series have been completed, a loopful was removed with the 0.05 ml. loop from the first well of the second row. Thus, by combining the dilutions in the two rows, a series consisting of 12 dilutions (1/2, 1/3, 1/4, 1/6, etc.) was obtained. Subsequently, 0.05 ml. of the serum to be absorbed and 0.05 ml. PBS were added to each dilution. The contents of the wells were mixed up with a rust-free wire, beginning at the highest virus dilution, and the titration trays were incubated at 37° C. for 30 min. Meanwhile the trays were shaken several times either gently mechanically or by a suitable vibrator.

After incubation a sample of 0.025 ml. was taken from each mixture for HA test.

\* (R) Labor MIM, Budapest.

Table 1. Data from a representative antiserum absorption test

Virus dilution Ha units per diluted sample $10^5$	Virus Hung/1/71					
	1/2	1/3	1/4	1/6	1/8	1/12
	$6.6 \times 10^4$	$5.0 \times 10^4$	$3.3 \times 10^4$	$2.5 \times 10^4$	$1.6 \times 10^4$	$1.2 \times 10^4$
Hung/92/71 serum absorbed with the above indicated virus dilution						
Residual Ha units per sample	3000	500	27	—	—	—
HI titre to:						
Hung/92/71	N.T.	N.T.	N.T.	64	96	256
Hung/1/71	N.T.	N.T.	N.T.	< 2	8	32
HK/1/68	N.T.	N.T.	N.T.	32	64	128
Eng/878/69	N.T.	N.T.	N.T.	< 2	6	24

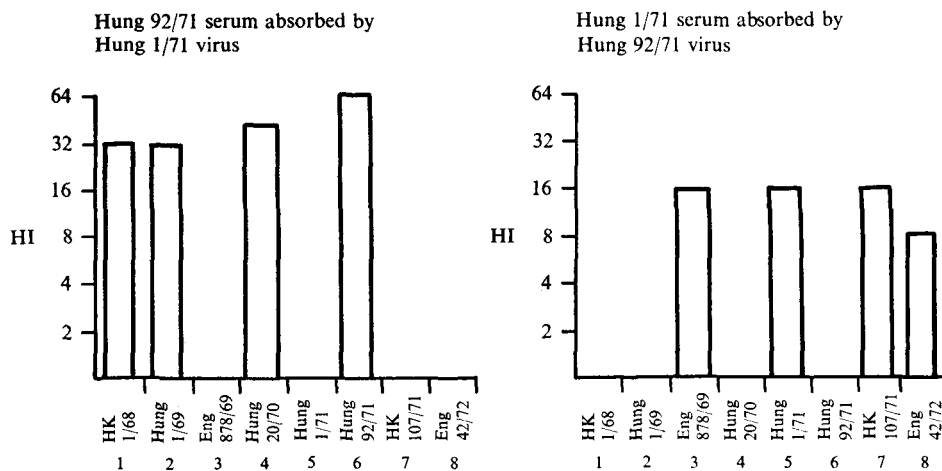


Fig. 1. Results of cross-absorption tests between influenza A strains Hung/92/71 and Hung/1/71

With the serum-virus mixtures containing no haemagglutinating virus, HI tests were carried out, using different virus strains. A representative serum-absorption test is shown in Table 1. In this experiment the 1/6 dilution was the first dilution showing no haemagglutination after serum absorption. In the same sample no HI antibody to the strain used for adsorption (Hung/1/71) could be detected, whereas the titre of the residual homologous antibody (to strain Hung/92/71) was 1/64, i.e. at least 32 times higher. The corresponding ratios from the tests carried out with serum samples obtained by adsorption with 1/8, 1/12 and 1/16 diluted virus suspensions were much lower: 12, 8 and 4, respectively, indicating that the absorption with the 1/6 diluted virus produced the most sensitive mixture for strain differentiation by simple HI titration. On the basis of such experiments, we call the first serum-virus mixture in which the HA test is negative optimally absorbed serum. For further strain differentiation larger volumes of absorbed samples of the optimal relative composition were produced.

## RESULTS

The antiserum absorption test as carried out according to the Microtitrator system has the great advantage that (i) very small volumes of concentrated virus are needed for the test and (ii) the results are independent of the virus and serum titre, i.e. standardized preparations are not needed.

Our method enabled us to follow the antigenic drift of the haemagglutinin of the influenza A (H3N2) virus by the analysis of the residual antibody in antisera optimally absorbed with different strains of the same main antigenic composition. We attempted to establish a gradient by arranging strains with those showing the most antigenic drift given the highest rank. Ranking of the strains has been based on our finding that if a serum is optimally absorbed with a strain standing higher on the gradient than that to which the serum is homologous the residual antibody

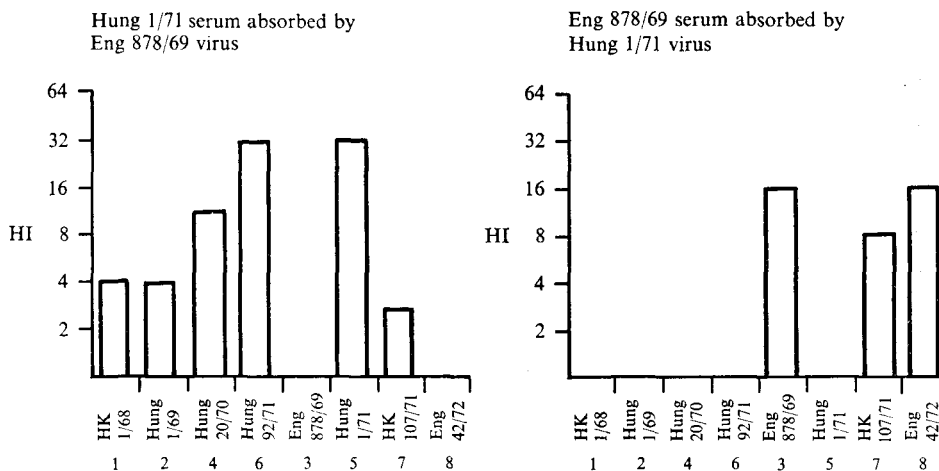


Fig. 2. Results of cross-absorption tests between influenza A strains England/878/69 and Hung/1/71

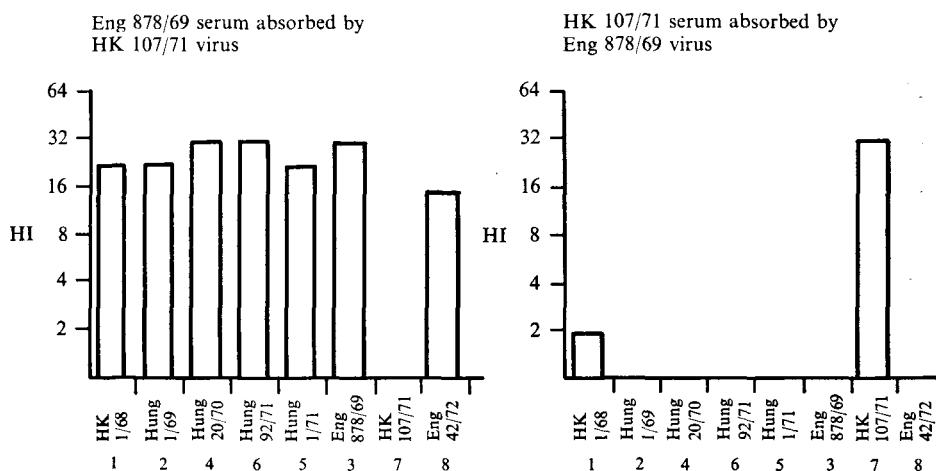


Fig. 3. Results of cross-absorption tests between influenza A strains England/878/69 and Hong Kong/107/71.

will react with the homologous strain, and also with all those standing lower on the gradient, but not with those standing higher and vice versa.

This principle is demonstrated in Fig. 1, illustrating the cross-absorption tests between strains Hung/92/71 and Hung/1/71. The optimally absorbed sera were examined with all the eight strains under study. The order of the strains in Figure 1 agrees with the chronological order of their isolation. According to the above principle, strain Hung/1/71 stands higher on the gradient than strain Hung/92/71, showing that its antigenic drift is more progressed. Even the England/878/69 strain should be placed higher than Hung/92/71.

The experiment shown in Figure 1 gives no information as to which of strains England/878/69 and Hung/1/71 stands higher on the gradient. In a further

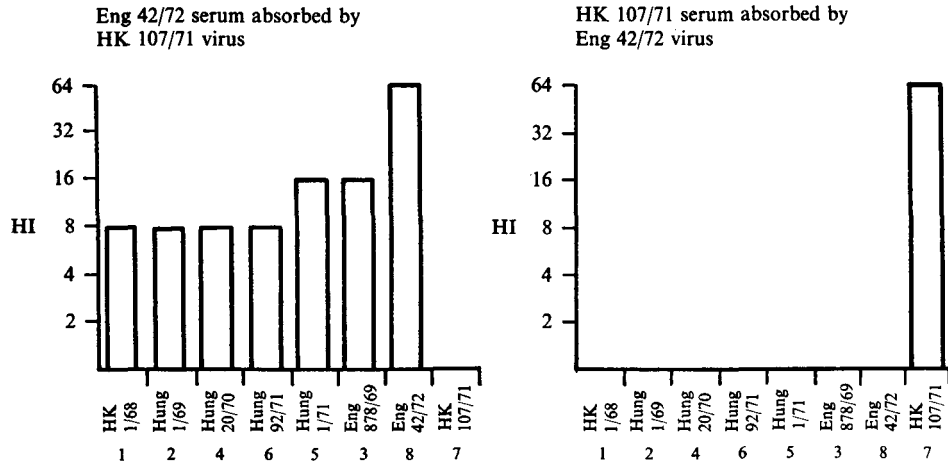


Fig. 4. Results of cross-absorption tests between influenza A strains England/42/72 and Hong Kong/107/71.

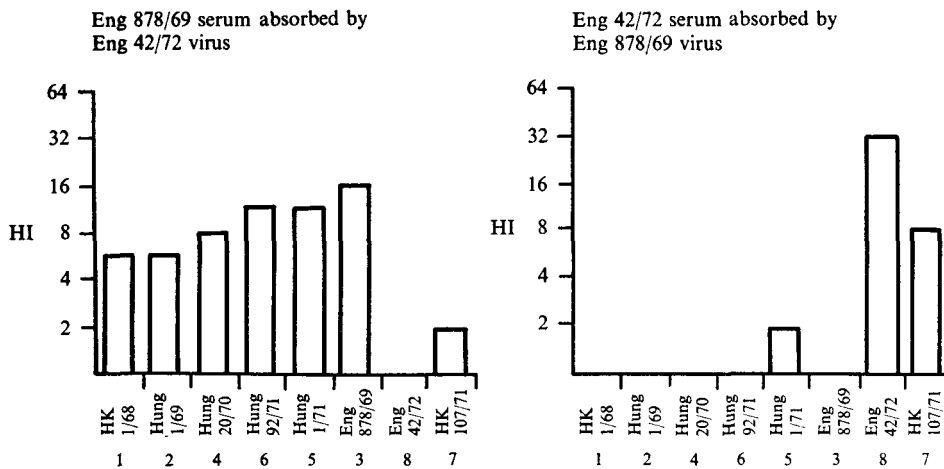


Fig. 5. Results of cross-absorption tests between influenza A strains England/878/69 and England/42/72.

experiment (Fig. 2) these two strains were subjected to the cross-absorption test. It is clear that England/878/69 stands higher. In the left graph of Fig. 2 the clear pattern is somewhat disturbed by the positive reaction of the residual antibody in serum Hung/1/71 with strain Hong Kong/107/71; the graph on the right side, however, clearly shows that the antigenic drift of the latter strain is more progressive. We return to this phenomenon in the Discussion.

Since the order of the Hungarian strains on the gradient had been established and it was proved by the experiments illustrated in Fig. 3, the relation to each other of the most progressive strains England/878/69, Hong Kong/107/71 and England/42/72 was examined (Figs. 3–5). Hong Kong/107/71 has proved to be the most progressive strain, followed by England/42/72 and England/878/69.

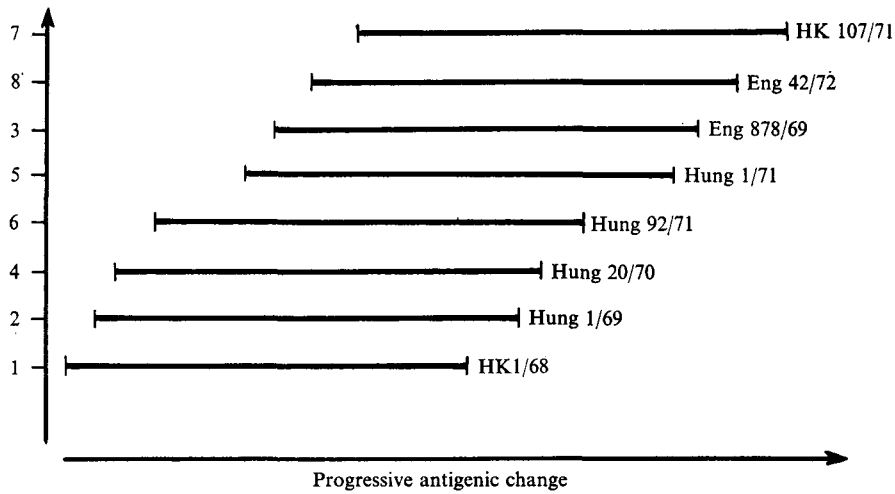


Fig. 6. Diagram showing the gradient from least to greatest antigenic drift of the eight strains of influenza A virus.

Accordingly, the gradient from least to greatest drift is as follows: Hong Kong/1/68, Hung/1/69, Hung/20/70, Hung/92/71, Hung/1/71, England/878/69, England/42/72 and Hong Kong/107/71 (Fig. 6).

#### DISCUSSION

The antigenic variation of the inter-pandemic antigen drift of human influenza A virus may be characterized as a step-by-step progressive change (Takátsy & Fürész, 1954; Takátsy & Fürész, 1957; Takátsy & Hamar, 1955) probably originating from spontaneous mutations. The selection of the mutants is governed by the population immunity. The role of antibodies in this process was proved in model experiments by Archetti & Horsfall (1950) and recently by Laver & Webster (1968), who characterized the antigenic drift by well-defined changes in the 'peptide map' of the virus. Since, if the propagation of influenza virus in the human population is not hindered by other factors, the antigenically most progressed strains have the greatest chance of causing the next epidemics, determination of the degree of this progress is of epidemiological importance. This character of the strains should be taken into consideration when strains for vaccine production are selected. The orientation in this field is greatly facilitated by the antiserum absorption test described in this paper.

Furthermore, it is clear from the present results that the anti-haemagglutinin molecules induced by any of the influenza A virus strains are heterogeneous. The antibodies demonstrable in an antiserum after absorption with different heterologous strains are sharply different from each other. It is not clear, however, whether these heterogeneous antibodies are induced by a heterogeneity of the haemagglutinins on the same strain or are a result of the heterogeneity of the immune response to the same antigen. We prefer the first alternative.

The antigenic drift of the haemagglutinin may be explained by serial spon-

taneous mutations. Similar mutants may arise and cause epidemics in different geographical areas and, if the population immunity in the different regions is similar, the antigenic drift may progress in parallel in remote countries. In some areas, however, more progressive mutation may occur than elsewhere and thus a strain may arise which has the greatest chance to cause an epidemic. In our opinion England/878/69, England/42/72 and Hong Kong/107/71 are such strains which called attention of the investigators using the cross HI test. The fact that in 1971 we isolated strains closely related to England/878/69 does not indicate that this strain had been introduced to Hungary. Since the Hungarian strains stand lower than England/878/69 on the gradient of antigenic drift, we suppose that they had developed independently and not by regression from a more progressed strain.

The Hong Kong/107/71 serum as absorbed by the less progressed England/878/69 virus did contain some antibody inhibiting the prototype strain Hong Kong/1/68. It is of interest that this single irregularity in our scheme occurred in relation to two strains isolated at different times in the same area.

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