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Host cell-mediated selection of influenza A (H3N2) virus variant subpopulations: lack of association between antigenic and receptor-binding properties

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SUMMARY

During the outbreak of influenza due to A (H3N3) viruses in Finland in 1985/6 virus pairs were isolated from the same clinical specimens in embryonated hens' eggs (CE) and in canine kidney cell cultures (MDCK). Some of these isolates, the E and M pairs, were distinguished by their reactions in haemagglutination inhibition (HI) tests carried out using polyclonal antisera, and by receptor-binding properties, as evidenced by differences in their elution activity from erythrocytes. Passage of the E- and M-virus isolates in the foreign host affected their serological characteristics, but the E virus did not convert to an M-like virus and the M virus did not convert to an E-like virus. Returning the viruses to grow in the host used for their isolation changed the serological reactions so that they were once more close to the reactions of the original isolates. This contrasts with the changes in receptor-binding properties. Rapid elution from hen erythrocytes, which has been described as a property of viruses binding to the SAα2,3Gal sequence in preference to SAα2,6Gal, characterized the virus passages grown solely in MDCK cell cultures. Cultivation of the M virus in CE, at any stage of its passage history, made the virus irreversibly incapable of elution. The M virus was more sensitive than the E virus to HI antibodies against heterologous viruses of the H3N2 subtype, and, when used as an antigen in HI serology, it more frequently (90% *vs.* 69%; $P < 0{\cdot}01$) detected diagnostic antibody responses in patients infected with viruses of this subtype in 1985/6. Use of antigens with a different passage history in HI serology provided evidence that this superiority, which may be due to the ability of the virus to pick out anamnestic antibody responses, is unrelated to the receptor-binding peculiarity of the M virus under consideration. The results support the concept that the host cell can select a diversity of virus variant subpopulations from a single clinical specimen during isolation and subsequent cultivation procedures. Moreover, the MDCK-grown influenza viruses may correspond better than the egg-grown isolates to the natural epidemic viruses.

INTRODUCTION

Antigenic differences between virus strains isolated from the same clinical specimen in embryonated hens' eggs (CE) and canine kidney (MDCK) cell cultures

have been detected for influenza B viruses (Schild *et al.* 1983) and also for H1N1 subtype (Oxford *et al.* 1987) and H3N2 subtype (Katz, Naeve & Webster, 1987; Pyhälä *et al.* 1987) influenza A viruses. Differential receptor-binding properties were proposed to be responsible for this host cell selection (Schild *et al.* 1983). This concept was supported by nucleotide sequence analysis of the haemagglutinin (HA) of MDCK cell-grown and CE-grown influenza B and H3N2 subtype influenza A viruses (Robertson *et al.* 1985, 1987; Katz, Naeve & Webster, 1987). In some instances at least, influenza viruses isolated in MDCK cells are obviously more akin in the antigenic structure of the HA to natural epidemic viruses than are the CE isolates from the same clinical source (Katz, Naeve & Webster, 1987; Oxford, 1987; Robertson *et al.* 1987). In accordance with this concept, MDCK cell-grown viruses used as antigens in HI serology have been shown to be superior to their CE-grown counterparts in detecting diagnostic antibody increases during influenza B virus (Lathey, Van Voris & Belshe, 1986) and H3N2 subtype influenza A virus infections (Pyhälä *et al.* 1987) as well as in detecting antibodies against H1N1 subtype viruses (Oxford *et al.* 1987).

In winter 1985/6, an outbreak of influenza due to H3N2 subtype viruses spread throughout Finland. Cultivation experiments were carried out with pairs of viruses isolated in CE and in MDCK cell cultures and originating from the same gargles. In the present paper attention is paid to antigenic characteristics and to a receptor-binding property of these viruses during their growth through successive passages in the two hosts. The association of these qualities with the ability of the viruses used as antigens in HI serology to detect diagnostic increases in antibody was also studied. It was established that there is no association between the antigenicity and the receptor specificity described in the present study.

MATERIAL AND METHODS

Viruses

The H3N2 subtype influenza A viruses were isolated during an outbreak of influenza in Finland that lasted from the end of December 1985 to April 1986. Virus pairs were isolated from the same gargles in embryonated hens' eggs (CE) and in Madin–Darby Canine Kidney (MDCK) cell cultures and subsequently passaged according to the principles followed during the previous outbreak in winter 1984/5 (Pyhälä, Pyhälä & Visakorpi, 1986). For isolation of virus in CE, 0·2 ml of gargle was inoculated into the amniotic cavities of 10- to 11-day–old CE. The allantoic and amniotic fluids were harvested and pooled after an incubation period of 3 days at 34 °C. To obtain further passages, the eggs were inoculated by the allantoic route with 0·2 ml of the virus diluted 10^{-3}; in the MDCK cell cultures the corresponding inoculum was 0·1 ml.

Serological tests and elution activity

HI tests were performed by a conventional microtechnique using 0·5% hen erythrocytes, four HA units of virus and sera pretreated with *Vibrio cholerae* filtrate (Pyhälä, Pyhälä & Visakorpi, 1986). Hyperimmune antisera used in antigenic analysis of viruses were produced by intraperitoneal injection of male

rats (Wistar, outbred) with virus-containing allantoic or tissue culture fluids as described previously (Pyhälä & Pyhälä, 1987). The results of the antigenic analysis are given in the dendrograms compiled as described by Beyer & Masurel (1985). Each dendrogram is based on geometric mean titres of HI tests performed in triplicate. Reference viruses were kindly provided by Dr J. Skehel (WHO Collaborating Centres for Reference and Research on Influenza, London).

To detect the elution activity of four HA units of virus from hen erythrocytes, the microtitration plates used for this purpose were incubated for 24 h at 37 °C before the final observation of the disagglutination pattern (Pyhälä, Pyhälä & Visakorpi, 1986). Complete disagglutination is indicated by + and complete agglutination by −.

Human sera

The initial sample consisted of acute and convalescent-phase sera sent for antibody testing to the National Public Health Institute, Helsinki, as part of the influenza surveillance programme. The sera were taken from military conscripts who had contracted an influenza-like illness during the 1985/6 epidemic season when H3N2 subtype viruses were circulating in the country. From this collection a set of 72 paired sera, showing ⩾ fourfold increase in complement-fixing (CF) antibodies to an influenza A virus, was compiled and studied for HI antibodies to the four virus antigens listed in Results. The CF tests were performed under the supervision of Dr Marjaana Kleemola, National Public Health Institute, Helsinki. Prior to the HI tests, the sera were pretreated with *Vibrio cholerae* filtrate at a dilution of 1 in 6.

RESULTS

Antigenic analysis and elution activity

In an antigenic analysis the influenza A (H3N2) strains isolated in CE in Finland in 1985/6 could be classified into two groups (Fig. 1). Group I was associated most closely with the reference strain A/Roma/6/85 whereas group II was related to A/Philippines/2/82, A/Hong Kong/1/84 and, more distantly, to A/Mississippi/1/85. In an additional test (data not shown), a number of viruses in the group II appeared to be similar to A/Leningrad/360/86.

Some virus strains were also isolated from the same gargles in MDCK cell cultures. Both types of isolate were available from six patients. Three of the CE isolates fell into group I and the other three into group II. The results of an antigenic analysis comparing these 12 viruses are given as a dendrogram in Fig. 2. The two groups listed in Fig. 1 and their association with the reference strains (the latter not included in the dendrogram) were verified with the six viruses isolated in CE. In contrast to this grouping, all six viruses isolated in MDCK were closely related to each other and fell into group II. As further demonstrated by Fig. 2, the elution activity of the viruses was strictly dependent on the host. Rapid elution from hen erythrocytes was characteristic of all MDCK isolates, whereas the CE isolates in both of the antigenic groups were incapable of elution.

One pair of viruses isolated from the same clinical specimen in CE and MDCK, A/Finland/25 E and M, was submitted to further examination. The M virus was

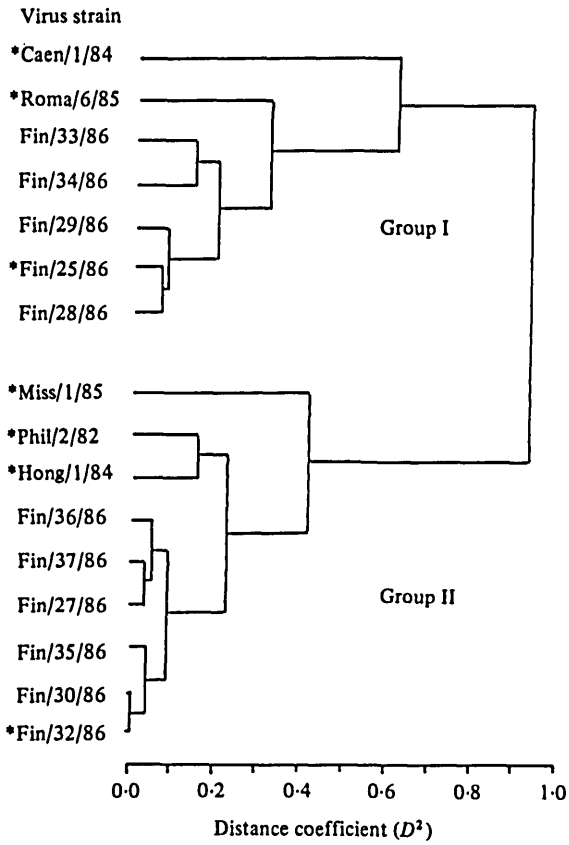

Fig. 1. Antigenic relationship between 5 reference strains of influenza A (H3N2) viruses and 11 field strains isolated in CE. Antisera against the viruses marked with an asterisk were used in HI tests.

characterized by higher sensitivity to antibodies against some heterologous viruses (Table 1). Both isolates were cultivated through successive passages in the host used for their isolation (E3-E6 and M3-M6, respectively). Further, the E virus was taken out to grow in MDCK cell cultures (E3M1-E3M2) and the M virus in CE (M3E1-M3E5). Results of an antigenic analysis comparing these 15 viruses (assortment A) are given as a dendrogram in Fig. 3. After 3-5 passages in the new host, the E and M viruses were returned to grow for 3 passages in their original isolation hosts. Results on an antigenic analysis of these new passages, together with eight early passages included for comparison, (assortment B) are given in Fig. 4. The assortments A and B were studied separately. The main findings concerned the various passages of the A/Finland/25/86 isolates are as follows.

(1) The antigenic properties of the E virus varied somewhat during subsequent passages in CE, but a more thorough conversion occurred immediately the virus was taken to grow in MDCK. Even then the virus could be clearly distinguished from the M virus. Returning the virus to grow in CE made it draw near to the original E-virus isolate once more.

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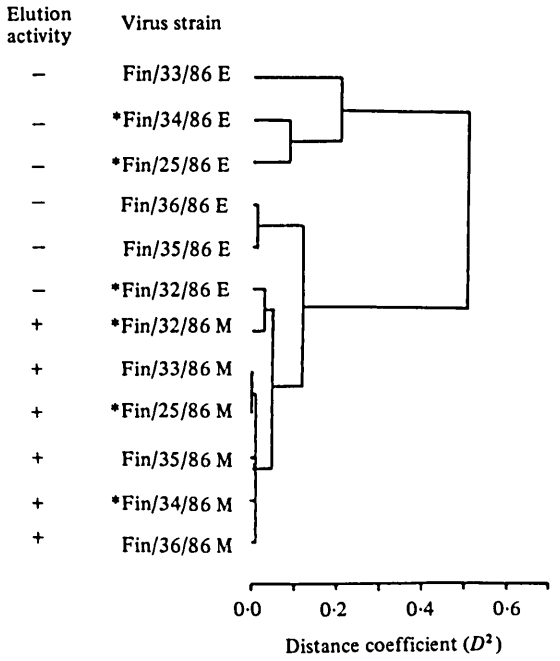

Fig. 2. Elution activity and antigenic relationship between six influenza A (H3N2) virus strains isolated in CE (E) and corresponding strains isolated from the same clinical specimens in MDCK cell cultures (M). Antisera against the viruses marked with an asterisk and against the five reference strains given in Fig. 1 were used in HI tests.

Table 1. *Sensitivity of the egg-grown (E) and MDCK-grown (M) isolates of influenza A/Finland/25/86 to HI antibody*

	HI titres ($\log_2$)		
Antisera against	E	M	Difference
A/Finland/25/86 E	8·9	7·6	1·3
A/Finland/25/86 M	3·9	7·9	−4·0
A/Philippines/2/82 E	3·6	8·9	−5·3
A/Hong Kong/1/84 E	5·6	7·2	−1·6
A/Caen/1/84 E	8·9	7·9	1·0
A/Mississippi/1/85 E	6·3	7·6	−1·3
A/Roma/6/85 E	6·9	7·9	−1·0

(2) The antigenic properties of the M virus remained stable during subsequent passages in MDCK. Conversion occurred when the M virus was removed to grow in CE. During further cultivation in CE a drift could be recorded, but the new passages still formed a comparable solid group separate from the E virus and its derivatives. As with the E virus, returning the M virus to grow in the original isolation host, now in MDCK, made it behave, in the antigenic analysis, very like the original isolate.

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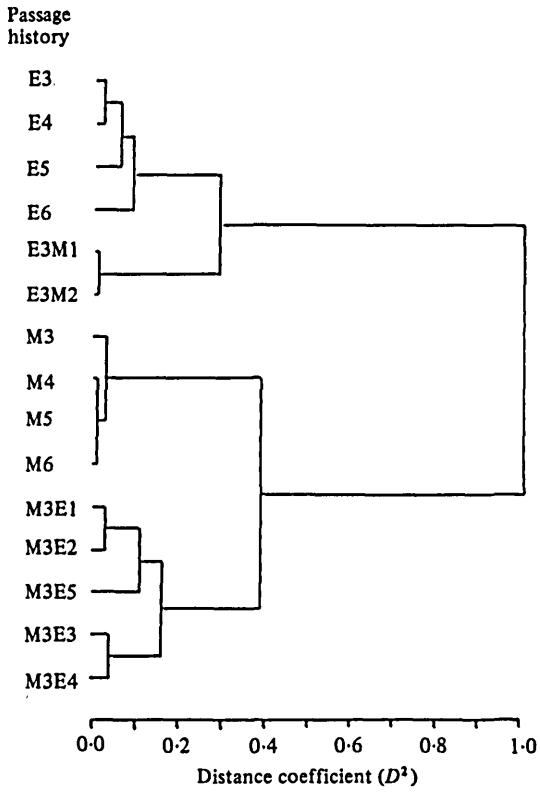

Fig. 3. Antigenic relationship between successive passages (assortment A) of influenza A/Finland/25/86 (H3N2) strains isolated from the same clinical specimen in CE and MDCK cell cultures. The 11 antisera of Fig. 2 were used in HI tests.

(3) As for elution activity, all virus passages which had been grown in CE at least once, at any stage in their passage history, appeared to be incapable of elution from hen erythrocytes. Viruses which had been isolated and subsequently passaged solely in MDCK, in fact passages M3-M6, eluted rapidly. This pattern could also be confirmed with a greater variety of virus passages originating from the E and M isolates of A/Finland/25/86 (Fig. 5).

In addition to A/Finland/25/86 E and M, two viruses, A/Finland/34/86 E and M, which had been isolated from another patient and also exhibited clear differences in the initial antigenic analysis (Fig. 2), were chosen for further study. The isolates were cultivated through the essential passages of those taken up in the case of A/Finland/25/86. Findings on the antigenic properties and elution activities of these derivatives (data not shown) were very similar to those obtained with the A/Finland/25/86 E and M viruses.

Elution activity was also studied with a number of passages produced from the E and M isolates of A/Finland/32, 33, 35 and 36/86. Three of these virus pairs consisted of E and M isolates which, in contrast to A/Finland/25/86 E and M, did not differ from each other in the antigenic analysis of Fig. 2 or differed no more than slightly. The findings on the elution activity of these passages were in all

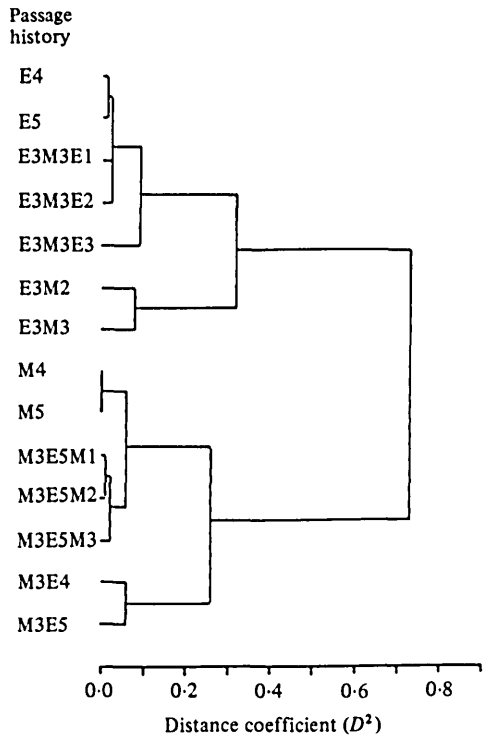

Fig. 4. Antigenic relationship between successive passages (assortment B) of influenza A/Finland/25/86 (H3N2). The 11 antisera of Fig. 2 were used in HI tests.

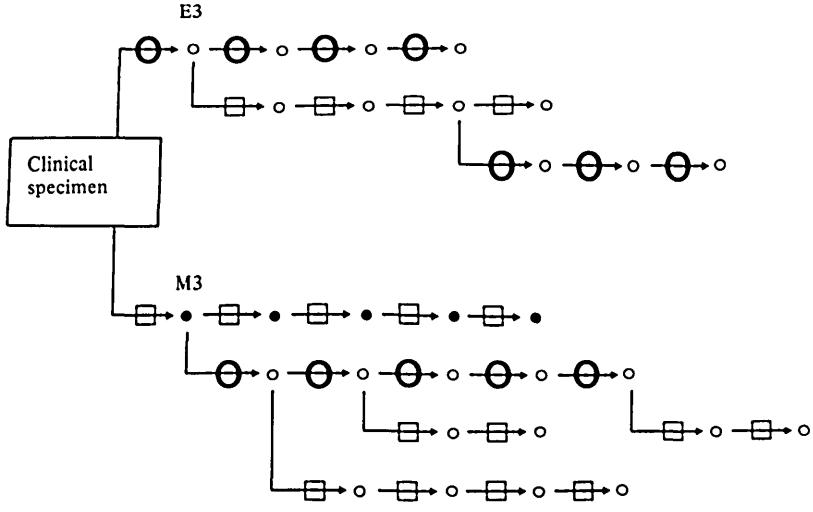

Fig. 5. Effect of cultivation in different hosts on elution activity (● = +, ○ = −) of egg-grown and MDCK-grown isolates of influenza A/Finland/25/86 (H3N2). Cultivation in: -o-, CE; -⊖-, MDCK.

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Table 2. *HI-antibody response to influenza A (H3N2) viruses in a sample of 72 patients exhibiting a diagnostic increase in CF antibody to influenza A in the season 1985/6*

Antigen in the HI test; virus strain, passage history and elution activity			HI-antibody response				
			⩾ four-fold increase in titre; number (%) of cases	titre of ⩾ 24 in acute phase; number (%) of cases	GMT of acute phase sera*	GMT of conval. phase sera*	× -fold increase in GMTs
A/Finland/25/86 E3	−		50 (69)	5 (7)	7·1	37·8	5·3 ×
A/Finland/25/86 M3	+		65 (90)	6 (8)	7·8	75·4	9·7 ×
A/Finland/25/86 M3E5M2	−		61 (85)	7 (10)	7·9	74·7	9·5 ×
A/Finland/31/80 E	−		67 (93)	19 (26)	10·9	133.1	12·2 ×

* A titre of < 12 is taken as 6.

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cases very similar to the findings on the A/Finland/25/86 virus derivatives given in Fig. 5. Thus, a single passage in CE was enough to render the MDCK-grown virus irreversibly incapable of elution from hen erythrocytes.

Serological diagnosis

The set of paired sera taken from patients infected with H3N2 subtype viruses during the 1985/6 outbreak was studied for HI anti-bodies against four virus antigens. Three of the viruses were representatives of different passages of A/Finland/25/86 (E3, M3 and M3E5M2). A/Finland/31/80 E was included as an antigenic variant of H3N2 subtype viruses from the near past (closely related to A/Bangkok/1/79), and was thus thought to be capable of measuring anamnestic antibody responses. Of the A/Finland/25/86 antigens used, the M3 virus detected diagnostic antibody increases more frequently than did the E3 virus (90% *vs.* 69%; $P < 0{\cdot}01$). Results for the M3E5M2 virus, which is incapable of eluting from erythrocytes, did not differ substantially from those for the M3 virus which was characterized by high elution activity. More data are given in Table 2. Attention is drawn to two points: (1) The antigen best able to detect significant antibody responses was the old virus strain, A/Finland/31/80 E. (2) The distribution of acute phase titres against the three A/Finland/25/86 virus passages differed from each other only slightly, whereas a higher acute phase antibody level was measured against A/Finland/31/80 E.

DISCUSSION

Cultivation of the E- and M-virus isolates in the foreign host modified their serological characteristics, but the E virus did not convert to an M-like virus, and the M virus did not convert to an E-like virus. This indicates that the differences between the E- and M-virus isolates might, to some extent but not exclusively, be due to host cell-dependent glycosylation of the haemagglutinin. The M-virus isolates were very similar to each other in their serological reactions whereas the E-virus isolates were antigenically heterogeneous. Attributed to host cell selection, this finding is consistent with results for H3N2 subtype viruses isolated in Memphis in 1985 (Katz, Naeve & Webster, 1987) and also with results for recent H1N1 subtype viruses (Oxford *et al.* 1987). Adaptation of virus mixtures, probably present in the original clinical specimen (Patterson & Oxford, 1986), to grow in CE may result in selection of virus subpopulations antigenically distinguishable from the viruses isolated in MDCK cell cultures. Further, variant viruses may arise, even in a uniform virus population, as a result of mutations during egg adaptation (Robertson *et al.* 1987).

The selection of antigenic properties demonstrated in the present study by using hyperimmune antisera might be prominent during virus isolation in CE. Such a powerful selective pressure for antigenic variants could not be demonstrated, however, when the M-virus isolates were subsequently passaged in CE. This does not exclude the possibility of demonstrating a strong host-cell selection, even in the CE passages, using proper monoclonal antibodies or different virus inocula. In any case, the lack of, or only modest, selection for antigenicity is different from results obtained with H1N1 subtype influenza A (Oxford *et al.* 1987) and influenza

B (Schild *et al.* 1983) viruses isolated in MDCK cell cultures. During subsequent passages in CE these viruses were shown to convert irreversibly to E-like phenotypes. As for the H3N2 subtype viruses studied by Katz, Naeve & Webster (1987), the subsequent growth in CE of virus isolated in MDCK cell cultures sometimes, but not invariably, let to the selection of an E-like virus population. There are many explanations for the lack of selection during cultivation of the H3N2 subtype M-virus isolates in CE. One is that, during isolation procedures, selection had occurred in a polymorphic virus population, not only in CE but, preferring other subpopulations, also in MDCK cell cultures. Indeed, Rott *et al.* (1984) showed that changes in HA of an egg-grown H3N2 virus strain occurred while it was adapting to growth in MDCK. Despite the possible lack of selection for antigenicity, strong selective pressure for a receptor-binding property was detected in the present study during growth of the M-virus isolates in CE.

The E- and M-virus isolates could be distinguished by their ability to elute from erythrocytes. Virus neuraminidase hydrolyses efficiently the SAα2,3Gal linkage of the receptor determinant in the cell surface sialyloligosaccharides (Carroll, Higa & Paulson, 1981). Rapid elution, which is a property of viruses binding to the SAα2,3Gal sequence in preference to SAα2,6Gal, characterized the M-virus isolates, whereas the E-virus isolates were incapable of elution. Of these two sequences, SAα2,6Gal is known to be the common receptor determinant for H3N2 subtype viruses isolated from human beings (Rogers & Paulson, 1983; Rogers *et al.* 1985). Strong selection was demonstrated when the M-virus isolate was subsequently cultivated in CE. Growth in CE, at any stage of the passage history, made the M virus incapable of elution, even when the virus was returned to grown in MDCK cell cultures once more.

Evidence has accumulated that the host cell selection of antigenically distinct subpopulations can be explained by differential receptor-binding properties (Robertson *et al.* 1987). In influenza B viruses, amino-acid substitution which altered the glycosylation of the HA molecule in close proximity to the receptor-binding pocket was associated with the adaptation of the virus to growth in CE (Robertson *et al.* 1985). The role of glycosylation in host-cell-binding properties has also been demonstrated with influenza A (H1N1) virus variants (Deom, Caton & Schulze, 1986). On the other hand, Katz, Naeve & Webster (1987) have shown that in H3N2 subtype viruses, amino acid substitution adjacent to the receptor-binding pocket but not directly affecting the glycosylation, might result in antigenic and biological differences between a virus grown in CE and its MDCK-grown counterpart. The break of association between the antigenic and receptor-binding properties detected in the present study can be interpreted to mean that, in this case, the molecular basis responsible for the exceptional receptor specificity of the M-virus isolates cannot be pleiotropically responsible for the serological characteristics of these viruses. It is possible, however, that other differences in receptor-binding properties also exist, some of which may be accompanied by differences in antigenic properties and may form a basis for selection of these characteristics. Indeed, differences in sensitivity to γ-inhibitors, α-macroglobulin present in high titres in non-immune horse and guinea-pig sera, were demonstrated between some of the various virus passages of the present study, although the E- and M-virus isolates themselves appeared to be sensitive (data not shown).

Detailed studies are in progress. A number of H3N2 virus variants with altered receptor-binding properties has been selected using anti-haemagglutinin monoclonal antibodies (Daniels *et al.* 1987; Underwood, Skehel & Wiley, 1987). It may be possible to select, even without any antibody pressure, numerous receptor-binding variants from the recent H3N2 subtype viruses while they are adapting to growth in the different host cell systems, CE in particular.

The ability of the M virus, as an antigen in HI serology, to detect increases in diagnostic antibody more efficiently than the E virus is consistent with findings made during the previous outbreak of influenza due to H3N2 subtype viruses (Pyhälä *et al* 1987) and with influenza B viruses (Lathey, van Voris & Belshe, 1986). In the present study, the superiority also applied to an M-virus derivative (M3E5M2) and was independent of the receptor specificities of the viruses demonstrated by elution activity. The M virus appeared to be more 'avid' than the E virus for HI antibodies against heterologous viruses. This suggests that the superiority of the M virus in detecting dianostic increases might be related to its improved ability to pick out anamnestic antibody responses. Despite the cross-reactivity, the acute phase titres against the M virus were low, being in fact lower than the titres against a representative of the older antigenic variants (A/Finland/31/80). This result is consistent with findings made during the previous outbreak (Pyhälä *et al.* 1987) that there is a good association between pre-epidemic antibody to an MDCK-grown epidemic virus and protection. The results also support the concept (Oxford 1987) that MDCK-grown influenza viruses may correspond better than egg-grown isolates to natural epidemic viruses.

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