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Egg-grown and tissue-culture-grown variants of influenza A (H3N2) virus with special attention to their use as antigens in seroepidemiology

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SUMMARY

A field strain of influenza A (H3N2) virus isolated in embryonated eggs during the 1984–5 influenza outbreak (A/Finland/13/85E) was compared in an antigenic analysis with virus from the same clinical specimen isolated in MDCK cell cultures (A/Finland/13/85M). The M-virus appeared to be more sensitive to haem-agglutination-inhibiting antibodies against heterologous viruses than did the E-virus. The results of propagation and plaque purification experiments support the hypothesis that a single clinical specimen may consist of distinct antigenic variant subpopulations promoted selectively by the host during isolation procedures. Receptor-binding properties are discussed as a possible explanation for this selectivity.

A set of 471 paired sera consisting of pre-epidemic and post-epidemic specimens taken from the same subjects in 1984-5 was studied for haemagglutination-inhibiting antibodies to six influenza A (H3N2) virus strains, including the E-virus and the M-virus from A/Finland/13/85. Of the antigens used, the M-virus detected significant antibody increases more frequently than did the E-virus (10·0 *v.* 5·9 %). The superiority of the M-virus may rest primarily in its ability to pick out anamnestic antibody responses. Irrespective of this cross-reactivity, pre-epidemic antibody to the M-virus was fairly well associated with protection. In the set of sera (230 specimens) collected in summer 1985 to represent different age groups, the antibody status against the M-virus was significantly better than the status against the E-virus. The results suggest that, at least in some instances, antibody to MDCK-grown virus is a more accurate indicator of the immune status of a community than antibodies to egg-grown virus variants.

INTRODUCTION

During the outbreak of influenza A (H3N2) viruses in Finland in 1984–5, an antigenic heterogeneity of field strains isolated in embryonated eggs was detected using polyclonal antisera in haemagglutination inhibition (HI) tests (Pyhälä, Pyhälä & Visakorpi, 1986). In addition to these egg-grown isolates, a number of virus strains were isolated from the same clinical specimens in MDCK (Madin–Darby canine kidney) cell cultures. Preliminary HI tests revealed

differences in antigenic properties between viruses isolated from the same source in the two hosts. One of these virus pairs, A/Finland/13/85E (isolation in eggs) and A/Finland/13/85M (isolation in MDCK), was chosen for further study.

An antigenic analysis performed using the HI test with monoclonal antibodies and polyclonal sera enabled Schild *et al.* (1983) to show that cultivation of influenza B virus in eggs selectively promotes subpopulations of antigenic variants which are different from the virus grown in MDCK cell cultures. In H3N2 subtype viruses of influenza A, an immunogold labelling technique allowed the identification of distinct antigenic variant subpopulations within a single isolate, and provided support for the hypothesis that·the host cell exerts selective pressure on subpopulations of viruses, resulting in the emergence of antigenic variants (Patterson & Oxford, 1986). In another recently published study on H3N2 subtype viruses (Styk *et al.* 1986), two stable virus lines from the same strain were demonstrated, using the HI test with monoclonal antibodies.

The molecular changes in the virion haemagglutinin responsible for the adaptation of the influenza B virus to growth in eggs have been documented, and the conclusion drawn that they affect the affinity of the virus for cellular receptors (Robertson *et al.* 1985). Host cell-mediated selection of mutant viruses with altered host cell binding properties has also been reported with H1N1 subtype influenza A viruses (Deom, Caton & Schulze, 1986). The present study provisionally considers selectivity exerted by the host, and receptor specificity of the H3N2 subtype viruses isolated in eggs and in MDCK cultures.

Influenza viruses adapted to growth in eggs have been routinely used as antigens in studies of immunity in man as well as in production of inactivated influenza vaccines. The results by Schild *et al.* (1983) raised the question of whether the exclusive use of egg-grown viruses as antigens for serologic diagnosis and seroepidemiology might give incomplete or even misleading results. Furthermore, antigenic differences between egg-grown viruses and viruses of infected persons can be proposed as possible contributory factors in the frequently observed inadequacy of protection resulting from influenza vaccinations. Indeed, tissue-culture-grown influenza B virus antigens were shown to be superior to egg-grown antigens in the detection of diagnostic antibody increases achieved with the HI test in patients infected with the influenza B virus (Turner *et al.* 1982; Lathey, Van Voris & Belshe, 1986). In the present study, egg-grown and MDCK-grown viruses from the same source were used as antigens and compared with each other in the seroepidemiology of influenza A (H3N2).

MATERIALS AND METHODS

Cultivation and plaque purification of virus

Viruses were isolated and propagated for further passage in embryonated hen's eggs and in Madin–Darby canine kidney (MDCK) cell cultures as previously reported (Pyhälä, Pyhälä & Visakorpi, 1986).

Single-plaque purification of the viruses was performed on monolayers of MDCK cells in plastic tissue culture dishes (30 mm diam.). The dishes were inoculated with 6×10^5 cells in 2 ml of Eagle's minimal essential medium (MEM) supplemented with 10 % fetal calf serum and penicillin–streptomycin according to

the principles described by Tobita & Kilbourne (1974). On the following day the plates were rinsed, and inoculated with 0·1 ml of virus. The inoculum was adjusted to produce only a few plaques per plate. After adsorption for 45 min at 34 °C each plate was covered with 2 ml of overlay medium comprising MEM, $NaHCO_3$ (0·18 %), fetal calf serum (2 %), agarose (0·6 %) and the antibiotics. Trypsin was omitted from the medium. Thus viruses with haemagglutinins not susceptible to proteolytic activation in MDCK cell cultures could remain undetected (Rott *et al.* 1984). After 3 days at 34 °C in a CO_2 (4·0 %) incubator, 1 ml of a second overlay medium supplemented with neutral red (35 μg per ml) was added. The plates were then reincubated for 2–3 h. The virus was harvested from solitary plaques only, using a disposable 1 ml syringe with a needle. Variations noted in the morphology of the individual plaques were not studied systematically. The isolated material was inoculated intra-allantoically into 10-11-day-old chick embryos with 0·1 ml of maintenance medium.

Serum collections

(1) Altogether 471 pairs of sera were selected as before (Pyhälä & Aho, 1975) from serial blood specimens taken from pregnant women. The sera were originally sent to our institute by antenatal clinics for Rh-antibody testing. The first sera were pre-epidemic specimens taken in October–December 1984. Post-epidemic sera were collected from the same subjects in May–June 1985. Laboratory findings (virus isolations and serological results) from the National Public Health Institute, Helsinki, were used to define the epidemic period.

(2) A set of 230 post-epidemic sera, taken in June–August 1985, consisted of acute-phase specimens. The majority were sent to our laboratory by general hospitals in different parts of the country, originally for routine serological screening of virus antibodies. The number of subjects in each of the age groups (Fig. 3) ranged from 35 to 51.

HI tests

The tests were performed using a conventional microtechnique with virus-containing allantoic and tissue culture fluids at room temperature (Kendal, Pereira & Skehal, 1982). The sera were pretreated with *Vibrio cholerae* filtrate at a dilution of 1 in 6 to remove non-specific inhibitors. The first collection of sera was tested for antibodies to A/Finland/13/85M (H3N2) isolated and further cultivated in MDCK cell cultures, and for antibodies to five egg-grown virus strains (see Table 2 for strains), which represented the H3N2 subtype variants epidemic in recent years. The second collection of sera was studied for antibodies to the egg-grown and the MDCK-grown variants of A/Finland/13/85.

Antigenic analysis of viruses was performed using the HI test with hyper-immune antisera produced by intraperitoneal injection of male rats as reported previously (Pyhälä & Pyhälä 1987). The results of this analysis are given in a dendrogram based on coefficient matrices compiled as presented by Beyer & Masurel (1985). Reference viruses were kindly provided by Dr J. Skehel (WHO Collaborating Centres for Reference and Research on Influenza, London). The sensitivity of viruses to γ-inhibitors was studied using HI with horse and guinea-pig sera (Pyhälä, Pyhälä & Visakorpi, 1986).
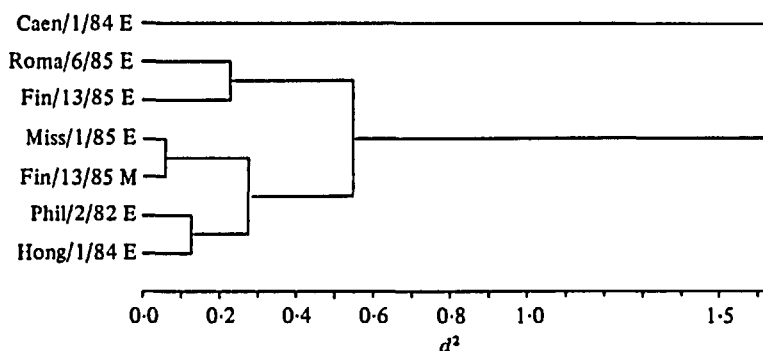
Fig. 1. Dendrogram illustrating the relation between the egg-grown and the MDCK-grown isolates of influenza A/Finland/13/85 (H3N2) and five reference strains. The dendrogram is based on geometric mean titres of HI tests performed in triplicate. Isolation and further passages in embryonated eggs (E) or in MDCK cell cultures (M).

Table 1. *Sensitivity of the egg-grown (E) and the MDCK-grown (M) isolates of influenza A/Finland/13/85 to HI antibody*

Antisera against:	HI titres ($\log_2$)		
	E	M	Difference
A/Finland/13/85E	7·9	8·6	0·7
A/Finland/13/85M	5·9	8·6	2·7
A/Philippines/2/82E	4·3	8·6	4·3
A/Hong Kong/1/84E	4·9	7·6	2·7
A/Caen/1/84E	7·2	8·2	1·0
A/Mississippi/1/85E	6·2	8·6	2·4
A/Roma/6/85E	6·6	8·6	2·0

Washed hen erythrocytes from individuals selected to give similar HA titres when tested with a set of reference viruses were used in the HI tests in a 0·5% suspension. The HI titres are expressed per 0·025 ml as a reciprocal of the highest dilution of sera causing inhibition of four HA units of virus.

RESULTS

Antigenic analysis

Antigenic relations between the egg-grown (E) and MDCK-grown (M) isolates of influenza A/Finland/13/85 (H3N2) and the reference strains representing epidemic serotypes of the H3N2 viruses were examined using the HI test with a panel of polyclonal rat antisera produced against each of the viruses. The results are given as a dendrogram based on taxonomic distance coefficients (d^2; Fig. 1). The E-virus was found to be most, but not very, closely related to A/Roma/6/85, whereas the M-virus corresponded well to A/Mississippi/1/85. A higher sensitivity to antibodies against heterologous viruses characterized the M-virus (Table 1). Both the E-virus and the M-virus isolates were sensitive to γ-inhibitors (a titre of ≥ 1536 with guinea-pig sera and a titre of 192–768 with horse sera).

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              ┌ P42 → E ●
              │ P43 → E ○   E ○ → E ◑ → E ◑
              │ P45 → E ○ ↗ M ○ → M ○ → M ○
              │ P47 → E ● ↘
              │ P48 → E ● ↗ E ● → E ● → E ●
              │ P50 → E ●   M ● → M ● → M ●
              │ P51 → E ●
              │ P53 → E ●
              │ P54 → E ○
        M ┤ P55 → E ●
           ↑  │ P56 → E ○
           │  │ P57 → E ●
           │  │ P58 → E ●
           │  │ P59 → E ●
           │  │ P60 → E ●
           │  │ P61 → E ○
           │  │ P62 → E ●
           │  │ P63 → E ○
           └ P64 → E ●

   E ● → E ● → E ● → E ● → E ●
 ↗                         ↘ M ● → M ● → M ○
□
 ↘
   M ○ → M ○ → M ○ → M ○ → M ○
                          ↘ E ●
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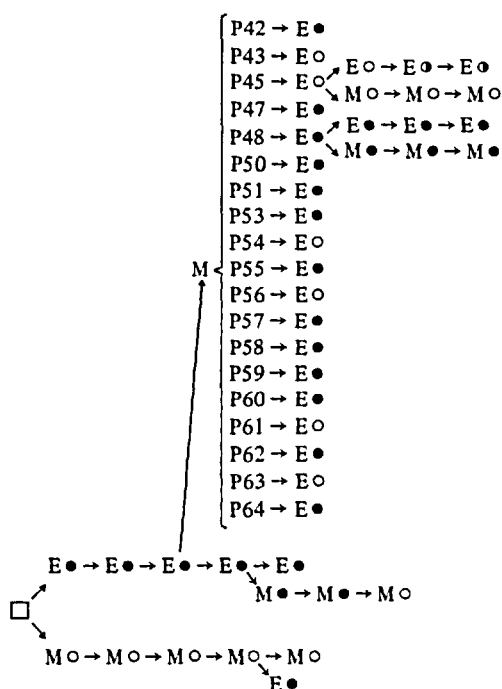
Fig. 2. Cultivation and plaque-purification measures of the egg-grown and the MDCK-grown isolates of influenza A/Finland/13/85 (H3N2): effect of the host on sensitivity to HI antibodies. □, throat washing; →, inoculation into host; E, embryonated egg as host; M, MDCK cell culture as host; ○, virus exhibiting 'high avidity'; ●, virus exhibiting 'low avidity'; P42–P64 indicates separate plaques.

Further cultivation and plaque purification

Stability of the sensitivity to heterologous antibodies was examined by propagation of the E-virus and M-virus isolates through further passages in the two hosts and by plaque purification (Fig. 2). In these experiments, a titre $\leqslant 48$ of anti-A/Philippines/2/82 antiserum was considered indicative of 'low avidity' and a titre $\geqslant 384$ indicative of 'high avidity'. The main results shown in Fig. 2 are as follows: (1) both the E-virus and the M-virus remained unchanged during additional passages in the host used in isolation; (2) a conversion in avidity occurred immediately the M-virus was removed to grow in eggs; (3) the E-virus also converted during cultivation in MDCK, but not as clearly and not until the third passage in this new host; (4) substrains exhibiting high avidity were frequently harvested from well-separated plaques of the E-virus of low avidity; (5) a substrain of low avidity remained stable during three additional passages in eggs and in MDCK, whereas a substrain of high avidity still had a tendency to convert towards a low-avidity virus in eggs.

Seroepidemiology

In the follow-up sample consisting of pre-epidemic and post-epidemic sera taken from the same individuals in 1984-5, the rate of serological infections was 11·3 % (53/471), as measured by a four-fold or greater increase in HI antibody titre to one or more of the six influenza A (H3N2) virus strains listed in Table 2. When

Table 2. *Serological infections in the follow-up sample of 1984-5 consisting of paired sera from 471 subjects*

Antigen in the HI test	Number (%) of serological infections
A/Finland/13/85E	28 (5·9)
A/Finland/13/85M	47 (10·0)
A/Finland/31/80E	38 (8·1)
A/Philippines/2/82E	28 (5·9)
A/Finland/1/85E	38 (8·1)
A/Mississippi/1/85E	41 (8·7)

Table 3. *Serological infections in the follow-up sample of 1984-5 in subjects grouped according to pre-epidemic antibody to A/Finland/13/85M*

Pre-epidemic HI titre	Number of subjects	Number (%) of serological infections	
		A*	B*
< 12	368	44 (12·0)	49 (13·3)
12	61	2 (3·3)	3 (4·9)
24	28	1 (3·6)	1 (3·6)
48	12	0	0
⩾ 96	2	0	0

* Significant increase in antibody titre detected with A/Finland/13/85M (A) or with one or more of the six viruses listed in Table 2 (B).
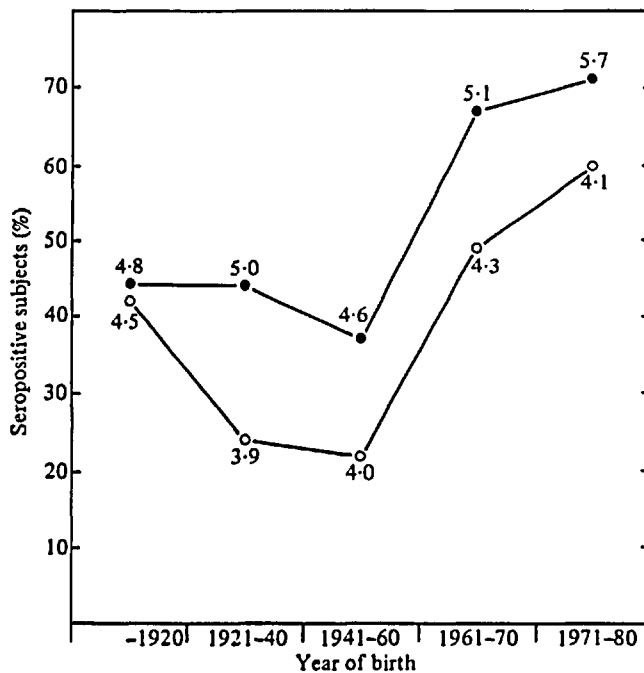

Fig. 3. HI antibody to influenza A/Finland/13/85E (○) and A/Finland/13/85M (●) in the set of 230 sera collected in summer 1985. Geometric means ($\log_2$) of the titres of seropositive subjects (HI titre ⩾ 12) are indicated by the figures next to the symbols.

measured with one antigen the rate varied, depending on the virus, from 5·9 % with A/Finland/13/85E to 10·0 % with A/Finland/13/85M. In all serological infections which could not be diagnosed using A/Finland/13/85M as antigen (six cases altogether), a twofold rise in this antibody was recorded. Table 3 lists the serological infections in the follow-up sample in relation to pre-epidemic antibody to A/Finland/13/85M. Forty-nine of the 53 infections were in seronegative persons, suggesting that the pre-existing anti-M-virus antibody correlated well with protection against infection.

In the set of sera collected for evaluation of immunity of the Finnish community in summer 1985 by means of HI antibody status, the antibody level was subsequently higher for the M-virus than for the E-virus in all but one of the age groups (Fig. 3). On the other hand, great differences between the age groups were recorded in the antibody status against both the E-virus and the M-virus.

DISCUSSION

The antigenic peculiarities of influenza A (H3N2) viruses isolated from the same throat-washing in embryonated eggs (A/Finland/13/85E) and in MDCK cell cultures (A/Finland/13/85M) may be, to some extent, host cell-mediated phenotypic properties arising from host antigen activity in the virus haem-agglutinin. Nevertheless, the cultivation and plaque purification measures support the hypothesis, which was presented concerning influenza B viruses (Schild *et al.* 1983), that a single clinical specimen may consist of heterogeneous viruses and that distinct antigenic variant subpopulations are selected by the host used for virus isolation. It can be proposed that cultivation in eggs favours the H3N2 virus subpopulation comparably non-sensitive to heterologous antibodies, whereas the subpopulation of sensitive virus may be somewhat superior in MDCK. During successive passages the dimorphism, obviously with altered proportions of the two sub-populations, seems to be maintained in both hosts. The antigenic characterization of the E-virus and the M-virus derivatives was carried out using the HI test and with polyclonal antisera alone. In these circumstances, antigenic heterogeneity may still exist among the viruses of 'high avidity' and among the viruses of 'low avidity'.

In investigations of influenza B viruses (Schild *et al.* 1983), the selectivity exerted by the host has been attributed to differences in receptor-binding properties. This hypothesis has been supported in studies demonstrating that adaptation of this virus to growth in eggs was associated with a change in one of the glycosylation sites of the virus haemagglutinin (Robertson *et al.* 1985). The role of glycosylation in host cell-binding properties has also bee demonstrated with influenza A (H1N1) virus variants (Deom, Caton & Schulze, 1986). As for the H3N2 subtype viruses, adaptation to growth in MDCK cell cultures has been reported to coincide with the introduction of several changes in the structure of the haemagglutinin (Rott *et al.* 1984). Some differences in receptor-binding properties have been detected between the E- and M-viruses of the present study (Pyhälä, Pyhälä & Visakorpi, 1986). Briefly, in contrast to the E-virus, the M-virus was incapable of agglutinating sheep erythrocytes and eluted rapidly from hen and human group-O erythrocytes. A rapid elution from erythrocytes has been

described as characteristic of Q-phase variants of H2N2 subtype human viruses (Choppin & Tamm, 1960) which, like Q-phase variants of H3N2 subtype human viruses (Rogers *et al.* 1983) as well as equine and avian isolates (Rogers & Paulson, 1983), bind to the $NA\alpha2,3$ Gal sequence rather than the $SA\alpha2,6$ Gal sequence as preferred receptor determinant in cell-surface sialyloligosaccharides (Carroll, Higa & Paulson, 1981). The rapid elution from erythrocytes is attributed to the virus neuraminidase, which efficiently hydrolysed only the $NA\alpha2,3$ Gal linkage (Carroll, Higa & Paulson, 1981). The molecular change that obviously determines the receptor specificity of the Q-phase variants of H3N2 subtype viruses has been described (Rogers *et al.* 1985). In contrast to the M-virus of the present study, the Q-phase variants are poorly reactive with HI antibody and in sensitive to γ-inhibitors (Choppin & Tamm, 1960). This difference suggests that, in spite of the similarity in elution rate, the two viruses are not identical in their receptor specificity.

The results of HI tests show that tissue-culture-grown influenza B virus antigens are superior to egg-grown antigens in the detection of significant increases in antibodies (Turner *et al.* 1982; Lathey, Van Voris & Belshe, 1986). A similar superiority was demonstrated in the present study with influenza A (H3N2). The M-virus appeared to be more avid to HI antibodies against heterologous viruses than did the E-virus. In these circumstances the superiority of the M-virus in detecting diagnostic increases in antibodies may be related to its improved ability to pick out anamnestic antibody responses, and not primarily or necessarily to its ability to recognize new antibody specificities. Irrespective of this cross-reactivity, serological infections were, with a few exceptions, restricted to subjects who were lacking measured levels of pre-epidemic antibody to the M-virus, even in circumstances where numerous closely related variants were concurrently circulating in the community (Pyhälä, Pyhälä & Visakorpi, 1986). The low protective titres of anti-M-virus antibody together with the high prevalence of this antibody in the sample collected in summer 1985 suggest that, at least in some instances, antibody to MDCK-grown virus is a more accurate indicator of the immune status of a community than are antibodies to egg-grown virus variants.

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