# Research
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# Antigenic heterogeneity within influenza A(H3N2) virus strains*

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*On the basis of their antigenic properties, influenza virus strains are classified into types and subtypes, which are further subdivided into variants that differ to various degrees in haemagglutination-inhibition assays. Evidence is presented that during infection with an influenza A(H3N2) virus the respiratory tract of a human patient often harbours more than one antigenic virus variant. These variants are frequently propagated by embryonated fowl eggs and monkey cells with different efficiencies, and this may lead to the selection of different variants by either of these host systems. Also, passage of virus by a given host is sometimes attended by changes in reactivity in haemagglutination-inhibition tests. In some cases the heterogeneity described also affects the specific immunogenicity of the virus in ferrets. Virus strains cloned in monkey kidney cell cultures gave variants that were stable upon further passage. These results may have implications for antigenic and biochemical investigations of epidemiologically relevant virus variants. It is argued that the antigenic drift of influenza A(H3N2) viruses is best characterized by analyses, both with post-infection ferret antisera and with panels of monoclonal antibodies, of virus strains isolated and passaged in monkey kidney cell cultures only.*

An important feature of influenza viruses is their antigenic and biological variability. Antigenic analyses reveal frequent, almost annual, minor changes in the haemagglutinin and neuraminidase antigens of epidemic strains within the subtypes of influenza A virus and within influenza B virus ("antigenic drift") (*1*).

Heterogeneity of RNA viruses is well known (*2*), and several reports on antigenic variability within

influenza virus strains have also appeared (*3–7*). In early studies, Burnet & Bull (*3*) observed small antigenic differences between virus isolated from the respiratory tract of an influenza patient and grown in the amniotic cavity of embryonated fowl eggs ("original" or "O" virus), and the same virus after adaptation to growth in the allantoic membrane ("derivative" or "D" virus). Antigenic divergency has also been observed upon passage within the same host system and been reported; for example, Hirst (*4*) detected antigenic differences between the allantoic harvests from different eggs inoculated with the same suspension of influenza A(H1N1) virus; Choppin & Tamm (*5*) between "substrains" of influenza A(H2N2) virus with divergent sensitivities to muco-protein inhibitors; and Kendal et al. (*6*) and Kilbourne

*(7)* between substrains of swine influenza virus.

All these reports dealt with small numbers of strains of influenza viruses and it could therefore not be excluded that the patients concerned had been infected simultaneously from epidemiologically different sources. However, also during routine diagnostic testing of influenza A and B virus isolates in haemagglutination-inhibition (HI) assays, we have frequently observed antigenic differences after different passages of the same strain, especially when virus grown in cell culture was compared with that grown in embryonated fowl eggs *(8)*. Similar antigenic variations have been noted after large-scale virus production for vaccine manufacture *(9)*. The selection of antigenic variants of influenza B virus brought about by passage in different host systems has also recently been described *(10, 11)*. Here we report on non-immune-mediated selection to influenza A(H3N2) viruses, describe the occurrence of such heterogeneity in clinical specimens, and discuss the possible consequences for the recognition of epidemiologically important virus strains.

### MATERIALS AND METHODS

#### Viruses and antibodies

Reference strains of influenza A(H3N2) virus that had been prepared in embryonated fowl eggs were obtained from Dr J. J. Skehel, WHO Collaborating Centre for Reference and Research on Influenza, Mill Hill, London, England. Virus strains from the Netherlands were isolated and subcultivated in tertiary cynomolgus monkey (*Macaca fascicularis*) kidney (MK) cell cultures, or in embryonated fowl eggs. Viruses from the United Kingdom, obtained from Dr M. S. Pereira, Central Public Health Laboratory, Colindale, London, England, had been isolated in baboon kidney cell cultures and passaged in embryonated fowl eggs. After inoculation, MK cells were maintained in tubes in roller drums at 33 °C in Eagle's minimum essential medium containing 0.5 mg of crystallized pig trypsin/litre.[a] When all cells showed cytopathic changes, the infected cell cultures were frozen at −70 °C. After thawing and low-speed centrifugation, the suspensions were used without further purification. Virus isolation and passage in eggs and antiserum preparation in ferrets were carried out according to standard methods *(12)*; however, in some cases, strain specificity was improved by adsorption with cross-reacting influenza virus *(9)*. The method for development of monoclonal-antibody preparations has been described previously *(13)*. Ascitic fluids were harvested and used

after purification over protein A *(14)* or hydroxyl-apatite *(15)* columns, unless otherwise indicated.

#### Inhibition tests

HI tests were performed with fowl erythrocytes using standard procedures *(12)* with minor modifications *(9)*. Ferret and rabbit antisera were pre-treated with cholera filtrate,[b] following the manufacturer's recommendations. The various virus preparations to be compared were allowed to react with the same panel of "master dilutions" of antisera (twofold dilutions) and monoclonal-antibody preparations (tenfold dilutions). HI titres were recorded as the reciprocal of the maximum dilution that caused complete inhibition. Neuraminidase-inhibition assays were performed according to standard procedures *(16)*.

#### Virus cloning by passage at terminal dilution

Serial twofold virus dilutions were inoculated onto 10 MK-cell tube cultures per dilution. After 10 and 20 days, the cultures were tested for virus replication using haemadsorption tests with guinea-pig erythrocytes. Cloning was considered successful if one of the dilutions showed virus growth in only one of 10 tubes, and all three subsequent dilutions were negative.

#### Heterogeneity studies with clinical specimens

Throat swabs were collected in the Netherlands in 1982 from four patients infected with influenza A(H3N2) virus and mailed in virus transport medium *(17)* to the Laboratory of Virology of the National Institute of Public Health and Environmental Hygiene, Bilthoven. Tenfold dilutions of the four samples were inoculated onto MK-cell tube cultures. Positive cultures were detected by screening for cytopathic effect, or, after 10 and 20 days by haemadsorption tests with guinea-pig erythrocytes *(12)*. Similar titrations were performed after neutralization of the specimen for 60 minutes at room temperature with an equal volume of monoclonal-antibody preparation in ascitic fluid that previously had been treated with cholera filtrate and which was used at a final dilution of 1:20. Also, tenfold dilutions of the untreated clinical samples were tested for infectivity in embryonated eggs. Amniotic preparations from apparently negative eggs were given two additional amniotic passages before finally being classified as negative.

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[a] Merck, Darmstadt, Federal Republic of Germany.

[b] Duphar, Weesp, Netherlands.

Table 1. Haemagglutination-inhibition (HI) assays with various passages from three influenza A(H3N2) virus strains

| Antigens used: | | | HI titres of: | | |
|---|---|---|---|---|---|
| Virus strain | Passage history | | F128[a] | F128A[b] | MA171[c] |
| A/Texas/1/77 | $E_{16}E_2$ | [1][d] | 768 | 512 | 24 000 |
| | $E_{16}MK_2$-A | [2] | 96 | <12 | <10 |
| | $E_{16}MK_2$-B | [3] | 192 | 128 | 16 000 |
| A/Bilthoven/1896/78 | $E_4$ | [4] | 512 | 512 | 24 000 |
| | $E_6$ | [5] | 48 | 12 | <10 |
| A/Bilthoven/20949/77 | $E_5$ | [6] | 64 | 24 | <20 |
| | $MK_2$ | [7] | 768 | 384 | 3 000 |
| | $MK_4$ | [8] | 256 | 12 | <20 |

[a] Post-infection ferret antiserum F128 was prepared with influenza A/England/321/77 virus grown in embryonated fowl eggs.

[b] F128A was obtained from F128 by cross-adsorption with influenza A/Texas/1/77 virus grown in MK cells.

[c] MA171, a monoclonal-antibody preparation against influenza A/Texas/1/77 virus haemagglutinin, was supplied by Dr G. C. Schild, London, England.

[d] Numbers in square brackets refer to individual virus preparations mentioned in the text.

## RESULTS

*Variation of influenza A(H3N2) virus reactivities in HI assays on passage*

Upon serial passages of influenza virus isolates at moderate dilutions, including a number of reference strains from the WHO Collaborating Centre for Reference and Research on Influenza, in either embryonated fowl eggs or MK-cell cultures, the reactivities of these viruses with antibody preparations in HI tests ("HI reactivities") often changed. Representative data on the antigenic characteristics of various passages for three influenza A(H3N2) virus strains (A/Texas/1/77, A/Bilthoven/1896/78, and A/Bilthoven/20949/77) are shown in Table 1. Upon receipt, influenza A/Texas/1/77 virus, which had been serially passaged 16 times in eggs ($E_{16}$) at the WHO Collaborating Centre for Reference and Research on Influenza, was serially subcultivated in eggs twice ($E_{18}$), and independently also serially passaged twice in MK-cell culture ($E_{16}MK_2$-A and $E_{16}MK_2$-B). The various virus harvests were examined in HI assays with ferret antiserum F128 that had been raised against egg-grown A/England/321/77 (H3N2) virus, obtained from the WHO Collaborating Centre for Reference and Research on Influenza, and which is closely related to A/Texas/1/77 virus. We routinely used this antiserum for identification of A/Texas/1/77-like viruses. Differences in serum titres ranged from two- to twelvefold within the same strain (Table 1). These same virus passages were, pairwise, more clearly distinguished by their reactivities with monoclonal-antibody preparation MA171

Table 2. Immunogenicity of haemagglutination-inhibition (HI) variants from the influenza A/Texas/1/77 virus preparations referred to in Table 1

| Virus preparation: | | | HI titres of: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus strain | Passage history | | F128 | F1[1][a] | F2[1] | F3[1] | F4[1] | F5[2] | F6[2] | F7[2] | F8[2] |
| A/Texas/1/77 | $E_{16}E_2$ | [1] | 768 | *1024*[b] | *1536* | *2048* | *3072* | 512 | 512 | 768 | 768 |
| | $E_{16}MK_2$-A | [2] | 96 | 96 (12)[c] | 256 (6) | 512 (4) | 768 (4) | *768* (1.5) | *1536* (3) | *2048* (3) | *2048* (3) |

[a] The notation F1[1] signifies that ferret antiserum F1 was made with virus preparation [1]. Other ferret antisera are designated analogously.

[b] Homologous titres are shown in italics. The homologous titre of antiserum F128 was 2048.

[c] Numbers in parentheses are ratios of homologous to heterologous titres.

(raised against A/Texas/1/77 haemagglutinin), which either reacted to high titres or did not react at all (Table 1).

The differences between the HI titres of F128 against virus preparations [1] and [2], [4] and [5], and [7] and [8], respectively, were enhanced by prior cross-adsorption of the antiserum with MK-cell-grown A/Texas/1/77 virus (F128A). This indicated that, for example, passages [1] and [2] reacted with different subpopulations of antibodies in the unadsorbed antiserum, which is consistent with a difference in their antigenic structures. In order to investigate the potential significance for vaccine development of the observed heterogeneity in antigenic structure, we infected two groups of four ferrets with preparation [1] and [2], respectively. HI assays with the resulting antisera against either of the viruses consistently showed clear differences in specific immunogenicity: the reactivity with the homologous preparations was 1.5 to 12 times higher than with the heterologous virus (Table 2). On the other hand, infection of ferrets with passages [6] or [7] resulted in each case in an antiserum that was more reactive with preparation [7] than with [6]. These two virus passages therefore differed in their "avidity" to both antisera (12), and the difference in reactivity of MA171 with the preparations [6] and [7] may also have arisen for this reason.

Differences in HI reactivity between passages were observed for many strains, and occurred at an average of one per 20 parallel subcultures prepared either in MK cells or in eggs. Comparison of egg-grown virus harvests with viruses derived from the same strain but cultivated in MK cells revealed differences in 98 of 135 (73%) influenza A(H3N2) viruses isolated since 1968. These 98 strains included 73 viruses from the Netherlands and the United Kingdom that were

isolated in MK cells, and 25 reference strains isolated in eggs and obtained as egg-grown passages from the WHO Collaborating Centre for Reference and Research on Influenza.

### Isolation of stable virus clones

When two MK-cell-grown preparations of influenza A/Bilthoven/935/75 (H3N2) virus (isolated and passaged in MK cells only), which were antigenically variable on further passage at routine dilutions and also had different HI reactivities, were subcultivated at terminal dilution in MK-cell cultures, two clones resulted that both retained the HI reactivities of their respective parental suspensions for over 100 parallel passages. Moreover, egg-grown virus preparations derived from both clones also exhibited the respective parental HI reactivities, and the clones were therefore considered antigenically stable on passage. The two clones produced approximately equal amounts of infectivity when grown in eggs or cultured in MK cells: $10^{7-8}EID_{50}$ and $10^{7-8}TCID_{50}$ per 0.1 ml, respectively. Similarly, a stable clone could be produced from each of the two other "variable" H3N2 virus strains isolated at Bilthoven that were passaged at terminal dilution.

### Heterogeneity of influenza A(H3N2) virus in clinical specimens

To investigate whether the virus variants detected in the individual laboratory preparations had already been encountered by patients, we performed re-isolation experiments on clinical specimens collected in 1982 in the Netherlands from four patients infected with influenza A(H3N2) virus (Tables 3 and 4). Previously, each of the four isolates obtained from these

Table 3. Occurrence of influenza A(H3N2) virus variants in clinical specimens from four patients in the Netherlands

| Clinical specimen | Neutralizing monoclonal antibody | Infectivity per 0.1 ml of clinical specimen titrated in: | | | Apparent frequency of minor variant[a] | |
| | | MK ($TCID_{50}$) | | Egg ($EID_{50}$) | | |
| | | No monoclonal antibody (I) | With monoclonal antibody (II) | No monoclonal antibody (III) | MK (II:I) | Egg (III:I) |
|---|---|---|---|---|---|---|
| S1 | MA3 | 3000 (1)[b] | 6 (2) | 3 (2) | 1:500 | 1:1000 |
| S2 | MA23 | 300 (3) | 2 (3) | 15 (4) | ≤1:150 | 1:20 |
| S3 | MA185[c] | 1000 (5) | 0.6 (5) | 2 (6) | ≤1:1600 | 1:500 |
| S4 | MA19 | 600 (7) | 10 (8) | 30 (8) | 1:60 | 1:20 |

[a] Minor variant isolated in MK cells after neutralization, and in eggs without neutralization, respectively.
[b] Numbers in parentheses denote patterns of reactivity in HI tests, as specified in Table 4.
[c] MA185, a monoclonal-antibody preparation against influenza A/Texas/1/77 virus haemagglutinin, was supplied by Dr G. C. Schild, London, England.

Table 4. Haemagglutination-inhibition (HI) reactivities of the virus variants referred to in Table 3

| Virus from clinical specimen | Pattern of HI | HI titres of ferret antisera to: | | | | | HI titres of monoclonal antibody to: | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | TEX[a] | | | BA1[b] | | BA2[c] | | ENG[d] | | | SHA[e] | PHI[f] | |
| | | TEX | BA1 | BA2 | ENG | PHI | 3 | 5 | 10 | 13 | 35 | 14 | 15 | 19 | 23 | 27 | 29 | 31 | 34 |
| S1 (MK) | (1)[g] | 320 | 80 | 160 | 320 | 480 | 1000 | 1000 | <10 | 1000 | 1000 | <10 | <10 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |
| S1 (egg) | (2) | 80 | 30 | 160 | 60 | 40 | <10 | <10 | <10 | <10 | 1000 | 1000 | 1000 | 100 | <10 | <10 | 100 | 1000 | 1000 |
| S2 (MK) | (3) | 320 | 80 | 320 | 640 | 1280 | 100 | 300 | <10 | 300 | 1000 | <10 | <10 | 1000 | 300 | 1000 | 300 | 1000 | 1000 |
| S2 (egg) | (4) | 120 | 20 | 80 | 80 | 320 | 1000 | 100 | <10 | <10 | <10 | <10 | <10 | 10 | <10 | 30 | 10 | 100 | 1000 |
| S3 (MK) | (5) | 160 | 80 | 320 | 320 | 640 | 1000 | 300 | <10 | 300 | 300 | <10 | <10 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |
| S3 (egg) | (6) | 160 | 40 | 80 | 160 | 160 | 1000 | 30 | <10 | <10 | 1000 | <10 | <10 | 1000 | 10 | 1000 | 100 | 1000 | 1000 |
| S4 (MK) | (7) | 480 | 80 | 320 | 320 | 640 | 1000 | 1000 | <10 | 1000 | 1000 | <10 | <10 | 1000 | 300 | 1000 | 1000 | 1000 | 1000 |
| S4 (egg) | (8) | 640 | 40 | 160 | 240 | 160 | 1000 | 1000 | 300 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | <10 | 1000 |
| Homologous titre | | 640 | 240 | 2560 | 1280 | 640 | 1000 | 1000 | 1000 | 1000 | 1000 | 10 000 | 10 000 | 1000 | 1000 | 1000 | 1000 | 1000 | 1000 |

[a] Influenza A/Texas/1/77 virus.
[b] A/Bangkok/1/79.
[c] A/Bangkok/2/79.
[d] A/England/496/80.
[e] A/Shanghai/31/80.
[f] A/Philippines/2/82.
[g] Figures in parentheses refer to the preparations, numbered as in Table 3.

specimens exhibited different HI reactivities, depending on whether eggs or MK cells had been used for isolation and cultivation (see below). The clinical specimens were next inoculated at various dilutions in MK-cell cultures, eggs, or MK-cell cultures after treatment with ascitic fluids containing one of the four different monoclonal-antibody preparations that were selected on the basis of their high reactivities in HI and neutralization tests with the original MK-cell-grown virus, but not with the egg-grown virus. All the virus harvests obtained were tested in HI assays against a panel of five ferret antisera and 13 purified monoclonal-antibody preparations (Table 4).

When tested in MK-cell cultures, the infectivity titres of the clinical samples varied between 300 and 3000 $TCID_{50}$ per 0.1 ml and dropped by 60-fold or more after neutralization with the respective monoclonal-antibody preparations (Table 3). No drop in the infectivity titre was observed when the four MK-cell-grown virus preparations were treated with ascitic fluids with antibodies to adenovirus. After treatment of specimens S1 and S4 with MA3 and MA19, respectively, viruses were isolated in MK-cell cultures that exhibited different reactivities from those isolated without such treatment. This result is consistent with the presence of antigenically different subpopulations in clinical samples in proportions of up to 1:60.

When titrated in eggs, the four clinical samples displayed infectivity titres that were 20- to 1000-fold lower than those found in MK-cell cultures (Table 3), and in all four cases the antigenic reactivity of the egg-grown virus was different from that of the corresponding MK-cell-grown virus. The viruses cultured in MK cells from specimens S1 and S4 after prior treatment with MA3 and MA19, respectively, had the same antigenic reactivity as the corresponding egg-grown viruses. Each of these variants had approximately equal infectivity titres in the two host systems, and this indicates that MK cells and eggs have about the same sensitivity to these minor antigenic variants; however, MK cells are more sensitive than eggs to the major virus variants isolated in MK cells without prior treatment with monoclonal antibodies.

## Characterization of influenza A(H3N2) virus strains from the Netherlands

During the winters of 1981–82 and 1982–83, 71 strains of influenza A(H3N2) virus were isolated in the Netherlands from different patients who were not epidemiologically connected. Eighteen arbitrarily selected isolates were propagated in MK-cell cultures as well as in embryonated eggs, and 53 isolates in MK cells only. The virus harvests were analysed in HI tests using a panel of five ferret antisera against WHO reference strains (A/Texas/1/77, A/Bangkok/

(a) HI assays with isolates grown in embryonated eggs
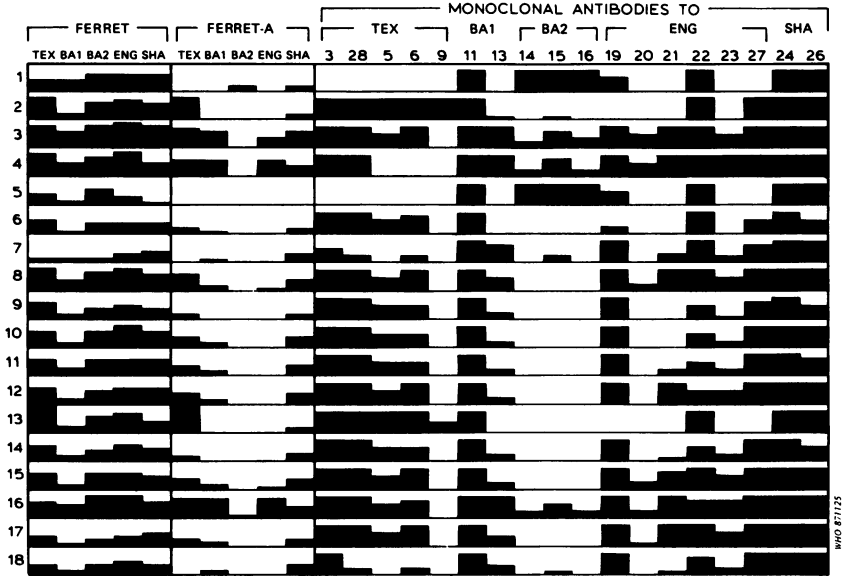


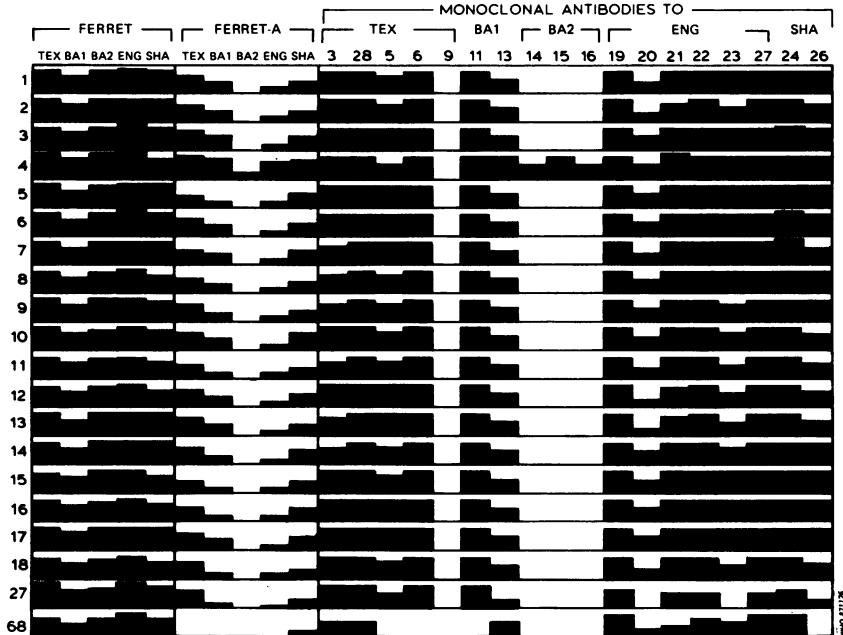(b) HI assays with isolates grown in MK cells



Fig. 1. HI assays with influenza A(H3N2) virus isolates from two epidemics in the Netherlands. The antiserum panel consisted of five ferret antisera (FERRET), the same antisera after cross-adsorption (FERRET-A), and 18 monoclonal-antibody preparations (numbered as shown) raised against influenza A(H3N2) virus A/Texas/1/77 (TEX), A/Bangkok/1/79 (BA1), A/Bangkok/2/79 (BA2), A/England/496/80 (ENG), or A/Shanghai/31/80 (SHA). Each HI titre is represented as a black bar, whose height corresponds to the logarithm of the titre.

1/79, A/Bangkok/2/79, A/England/496/80, and A/ Shanghai/31/80), the same five antisera after cross-adsorption, and 18 monoclonal antibodies raised against the same reference strains (Fig. 1). Among the 18 egg-grown virus preparations, nine different HI patterns could be distinguished (Fig. 1a), whereas among the 71 MK-cell-grown viruses only four profiles emerged (Fig. 1b). The main pattern in Fig. 1b was shown by 62 strains, while seven isolates, including strain 4, displayed another profile, as brought out by MA14, MA15, and MA16. Two other strains (27 and 68 in Fig. 1b) were distinguished by other monoclonal antibodies. Interestingly, the deviant pattern of isolate 4 was identical to that of the majority of strains obtained during the previous winter of 1980–81.

## DISCUSSION

The HI reactivity of the majority of the 135 influenza A(H3N2) virus isolates examined changed upon serial passages in embryonated eggs or MK cell cultures. Cloning of "variable" strains made them stable on further subcultivation in either host system. The uncloned virus preparations should therefore be considered as mixtures of virus variants, each of which is itself antigenically stable. Apparently, the proportions of these variants can change during a single passage to such an extent that different HI reactions result; however, preliminary studies on the reactivities in neuraminidase-inhibition tests have not shown a variability upon passage.

The results of both immunogenicity studies and cross-adsorption experiments with ferret antisera indicated that in some cases differences in antigenic structure, while in others differences in avidity, were involved (Tables 1 and 2). Possibly, in some instances differences in HI reactivity reflected those in receptor-binding specificities (18). In order to further clarify the genetic basis of the heterogeneity, we are currently carrying out nucleotide sequence studies on the haemagglutinin genes of the variants.

The variants observed within a given strain of influenza virus must have already been either present in the patients or generated in the laboratory during the isolation and passage procedures. The results for the clinical specimens from four patients reported here indicate that each of the latter had harboured at least two antigenically different variants (Table 3). This, together with the observation that virus-cloning procedures consistently yielded stable clones, favours the first of the above explanations. However, whether these variants were already present in the virus "inoculum" that infected the patient and had grown independently "side by side" in the host or had

developed in the patient as mutant viruses from a single variant remains a matter of speculation.

The ratios of infectivity titres between the various variants depend on the host system involved. Neutralization experiments showed that in the clinical samples the "major" variant had at least a 60-fold higher infectivity titre than the "minor" variant when titrated in MK-cell cultures. Unfortunately, it was not possible to determine the epidemiologically more relevant ratios for the tissues of the human respiratory tract. It cannot be excluded therefore that the major variant obtained from the MK cells represented only a minor variant in the patient, even when, in contrast to egg-grown variants, it was present in the vast majority of the patients infected with the viruses shown in Fig. 1.

Studies with monoclonal antibodies have indicated that in cloned influenza virus preparations mutants with an antigenically variant haemagglutinin occur at a frequency of $10^{-5}$ to $10^{-6}$ (19). Such mutants, in general, are not of epidemiological importance, since in aerosols the minimum infective dose of influenza A virus for humans is about 10 $TCID_{50}$ (20). In contrast, the presence of antigenic variants at frequencies of $10^{-1}$ to $10^{-3}$ in virus shed by patients, as reported here, may enhance the chance of successful infection in a population with immune defences that have different antigenic specificities (21). On the other hand, the relative frequencies of the various antigenic variants in a human host may vary with changing immunological pressure, and over a period of years such changes may account for the so-called antigenic drift of the influenza virus.

Variability of HI reactivity is of considerable significance in studies of influenza virus epidemiology, and the course of the antigenic drift might best be described in terms of all the variants identified in virus isolates. In the light of the present study, it seems appropriate for epidemiological purposes to pay most attention to the variants that grow readily in MK-cell cultures but poorly in eggs, since these appeared to represent the major antigenic variants in the clinical samples tested. Such variants should be defined by their reactivity with a panel of monoclonal antibodies. However, they should also be assayed against post-infection ferret antisera, because only then can it be concluded whether a given variant represents a new step in the process of antigenic drift.

Using monoclonal antibodies and ferret antisera, we are currently analysing MK-cell-grown virus isolates that did not subsequently undergo egg passage, in order to describe the various epidemics of influenza A(H3N2) virus since 1977 in terms of major and minor virus variants. Attempts to perform such a characterization with egg-grown virus isolates are frustrated by the large number of variants that occur in the same epidemic, even in a single country

(22) (Fig. 1) or town (23). Nevertheless, the existence of these variants is of interest in emphasizing that antigenic drift occurs in advance of new variants becoming the dominant population.

Analyses of older and newer strains propagated in MK cells will probably not yield data fundamentally different from those obtained in earlier studies with egg-grown viruses (1). For instance, the "evolutionary trees" constructed using the results of such studies for influenza subtype A(H1N1) virus (24), subtype A(H3N2) virus (25), and type B virus (26) are based on the gradual accumulation over time of mutations which may be largely identical for variants that, pairwise, are isolated from the same patients. This common variant history, which implies an identical immunological pressure, is also most likely to cause a sequence of changes in MK-cell-grown viruses that resembles the antigenic drift found for egg-grown viruses.

In view of the data we have reported here, it can be concluded that in order to compare two strains antigenically or biochemically in detail, it is essential to use several clones isolated independently from different substrates, e.g., MK cells and eggs. Finally, the heterogeneity we have described may have implications for the composition of influenza vaccines, since, despite the limited number of virus passages during vaccine manufacture, antigenic changes sometimes develop (9). However, the role of this heterogeneity during infection of vaccinated or unvaccinated individuals is not currently understood and requires further study.

## RÉSUMÉ

### HÉTÉROGÉNÉITÉ ANTIGÉNIQUE DES SOUCHES DE VIRUS GRIPPAL A(H3N2)

Pour les besoins des études épidémiologiques, les isolements de virus grippal sont couramment caractérisés par des réactions d'inhibition de l'hémagglutination, et il est en général implicitement admis que le virus testé est identique du point de vue antigénique à celui qui infectait le malade. Toutefois, nous avons souvent observé des modifications antigéniques chez les virus grippaux A et B, surtout chez les virus cultivés sur cellules rénales de singe et sur œufs de poules embryonnés. Lors de la présente étude, 73 % des 135 souches de virus grippal A (H3N2) isolées après 1968 aux Pays-Bas et au Royaume-Uni, ou provenant du Centre collaborateur OMS de Référence et de Recherche pour la grippe (Londres, Angleterre), ont montré de telles modifications. Ces différences ont pu être mises en évidence par inhibition de l'hémagglutination avec des immunsérums de post-infection de furets, des immunsérums ayant subi une adsorption croisée et, plus nettement encore, avec des préparations d'anticorps monoclonaux. Dans certains cas, l'hétérogénéité observée avait une incidence sur l'immunogénicité spécifique du virus, et dans d'autres, sur son avidité.

Le passage de ces souches "variables" à la dilution finale a donné des clones viraux qui se sont révélés stables sur le plan antigénique dans les passages ultérieurs, pour les quatre cas testés. Cela indique que les souches variables sont des mélanges de variants stables, dont les proportions peuvent changer au cours d'un passage, surtout lorsqu'on utilise un hôte différent. Il y a tout lieu de croire que l'hétérogénéité antigénique observée reflète la présence de différents variants viraux dans les voies respiratoires d'un même malade. Il est donc concevable que l'hétérogénéité décrite favorise dans une population humaine l'infection de sujets dont les défenses immunitaires ont des spécificités antigéniques différentes. A long terme, cette hétérogénéité peut faciliter la progression du glissement antigénique.

Les phénomènes rapportés ici doivent être envisagés en relation avec la caractérisation antigénique et biochimique de variants viraux d'importance épidémiologique, par exemple dans le cadre de la mise au point de vaccins. Il semblerait en conclusion que la meilleure description du glissement antigénique des virus grippaux A(H3N2) se fasse à partir des résultats d'analyses effectuées avec de l'immunsérum de furet et des préparations d'anticorps monoclonaux, sur des souches virales isolées et passées exclusivement sur cellules rénales de singes. Quant aux variants révélés dans les cultures sur œufs, ils gardent leur importance pour l'étude du mécanisme de glissement antigénique.

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