Influenza A virus haemagglutinin polymorphism: pleiotropic antigenic variants of A/Shanghai/11/87 (H3N2) virus selected as high yield reassortants

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Introduction

Influenza viruses are an outstanding example of viral polymorphism. The genetic non-homogeneity of standard laboratory strains (reviewed by Kilbourne, 1978, 1987), recent isolates (Robertson et al., 1991), or even unpassaged clinical material (Katz & Robertson, 1992), is increasingly recognized. For the most part, intrastrain differences are minor and detectable only through analysis with monoclonal antibody (MAb) panels. However, antigenic differences demonstrated using polyclonal antibodies in hyperimmune sera have been described (Kilbourne, 1978; Both et al., 1983; Johansson & Kilbourne, 1992). Such differences have potential significance in the perennial fabrication of high yield (hy) influenza virus reassortants (Kilbourne, 1969; Baez et al., 1980) for use in vaccines against antigenic variants emerging in nature.

We describe here two hy reassortants (X-99 and X-99a) derived from different subpopulations of A/Shanghai/11/87 (H3N2) that differ in antigenicity, binding affinity and yield. These differences introduced a dilemma in vaccine choice in 1989 because X-99a, the highest yielding reassortant, when subjected to initial antigenic analysis with ferret antisera appeared to be less broadly immunogenic. We shall show that both reassortants, although antigenically and biologically different, were equally immunogenic and protective in BALB/c mice to challenge by parental wild-type virus. Differences in HA phenotype were related to a Ser to Ile change at amino acid position 186. These findings emphasize the polymorphism of influenza virus strains as well as the need for caution in selection of vaccine strains from among antigenically distinct viral subpopulations.

Methods

Viruses. The hy reassortant viruses X-99 and X-99a were produced by genetic reassortment of A/Shanghai/11/87 (H3N2) with A/PR/8/34 (H1N1) influenza A virus variants as described under Results. Hy reassortants X-91, X-97 and X-101 were derived from reassortment of A/PR/8/34 with A/Leningrad/360/86, A/Sichuan/2/87 and A/Beijing 4/89 H3N2 viruses, respectively (Table 1).

Antisera. Antisera were produced by intravenous (i.v.) injection of rabbits with 3000 haemagglutinating units of purified virus. The rabbits were bled 42 days after initial injection and 7 days after a booster injection of virus on day 42. Prior to use in neutralization and haemagglutination inhibition (HI) tests, antisera were heated at 56 °C for 30 min and treated with Vibrio cholerae receptor-destroying enzyme as previously described (Kilbourne et al., 1990).

Serological titrations and antigenic analyses. HI, neuraminidase inhibition and neutralization tests and ELISA were carried out as previously described (Kilbourne et al., 1990). HI tests for antigenic analysis were performed in tubes using large volume transfers and interpolated dilutions. This precise method has a mean s.d. of ± 19% (Horsfall & Tam, 1953). Antibody titre ratios were calculated by the method of Archetti & Horsfall (1950). Homologous and heterologous
Table 1. Biological phenotype of A/Shanghai/11/87
HA mutants and their reassortants

<table>
<thead>
<tr>
<th>Virus</th>
<th>HA titre*</th>
<th>HI titre†</th>
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<tbody>
<tr>
<td></td>
<td>Egg</td>
<td>MDCK</td>
</tr>
<tr>
<td>A/Sh/87 (ly)</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>A/Sh/87 (hy)</td>
<td>126</td>
<td>45</td>
</tr>
<tr>
<td>X-99</td>
<td>256</td>
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</tr>
<tr>
<td>X-99a</td>
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<tr>
<td>X-99I–§</td>
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<td>8</td>
</tr>
<tr>
<td>X-99aE‖</td>
<td>1264</td>
<td>–</td>
</tr>
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</table>

* The reciprocal of endpoint dilution. Geometric mean of six individual eggs.
† Expressed as log$_{10}$ of value. NHS, heated at 56 °C, as the source of non-specific α2-macroglobulin inhibitor.
§ Inhibitor resistant escape mutant from the passage of X-99 with NHS.

PAGE. The differential migration of isolated virion proteins was studied by the method of Ritchey et al. (1977) using 7 to 14% gels under reducing conditions.

RNA sequencing. RNA sequence analysis was performed by the dideoxynucleotide chain termination method, using synthetic oligo-deoxynucleotide primers and reverse transcriptase essentially as described elsewhere (Cox et al., 1988) except that 10 μCi of [α-32P]dATP (Amersham; specific activity > 1000Ci/mmol) for each 5.5 μl of reaction mixtures was used and reverse transcriptase incubations and chase were done at 42 °C for 20 min. The HA1 domains of the HA genes of the X-99aE and Sh/ly (low yield) viruses (Table 1) were amplified by the PCR method (Saiki et al., 1988; X. Xu et al., unpublished) with primer 7, 5'd ACTATCATTTGCTTTTG as the forward primer and reverse complement primer 1184, 5'd ATGGCTGCTTGAGTGCTT as the reverse primer. Primers complementary to the mRNA sense strand 5'd CCTGCGATTCGGCGGGAAT, 5'd CGATATGTCTCTCCTGGTTC, 5'd TGGCATAGTCCAGTGCAG and 5'd TAAGGGTAAACGTTGCTG beginning at nucleotides 1090, 809, 588 and 379, respectively, were used for sequencing the asymmetrically amplified PCR products.

Production of X-99a HA-specific MAb. Female BALB/c mice (Charles River) were immunized with 25 μg of sucrose-gradient purified X-99a virus by intraperitoneal injection. Animals were immunized on two occasions, 3 weeks apart, rested for 10 weeks and boosted with 10 μg X-99a intravenous injections, 3 days before fusion. The fusions were performed by a standard method (Holmdahl et al., 1989) and positive cultures were cloned by limiting dilution.

Screening of MABs by immunostaining. The method of Usuba et al. (1990) was used. Briefly, MDCK cells were infected with virus and, following fixation with paraformaldehyde, hybridoma supernatants were added to each well. Binding was identified with peroxidase-linked goat antiguine IgG and the substrate 3-amino-9-ethyl carbazole. Hybridomas positive on X-99a but not PR8 or X-99 were considered X-99a-specific, and this was confirmed by HI testing.

Infection of mice. Groups of 20 g female BALB/mice were infected intranasally under light ether anaesthesia (Johansson & Kilbourne, 1991) and pulmonary samples of virus were measured as described previously (Schulman & Kilbourne, 1963).

Results

Derivation of X-99 and X-99a by reassortants from A/Shanghai/11/87 (H3N2) influenza virus

X-99 was produced by reassortment of A/Shanghai/11/87 (Sh/87) and A/PR/8/34 (PR8) viruses by the usual procedure of dual infection of a chick embryo allantoic sac (Kilbourne et al., 1971). Viruses containing the HA and neuraminidase (NA) antigens of Sh/87 virus were isolated by passage with PR8 antibody. When X-99 proved relatively poor yielding in vaccine production, we screened 26 eggs inoculated with Sh/87 virus to obtain the highest yielding wild-type virus, then carried out a second reassortment experiment with the hy mutant and PR8. The reassortant recovered (X-99a) consistently produced two to three times more HA than X-99. Like its wild-type parent X-99a differed in other biological properties from X-99, including binding affinity to antibody and non-specific inhibitor (see below). Furthermore, preliminary testing of X-99 and X-99a with specific ferret antisera at the Centers for Disease Control, Atlanta suggested that there were significant antigenic differences, with X-99a being less like contemporary H3N2 isolates and hence less suitable as a vaccine candidate. Repetition of reciprocal HI titrations with the same ferret sera in the Mount Sinai laboratory confirmed the antigenic difference in the reassortants, which by our quantitative analysis (Kilbourne et al., 1990) was 65% (data not shown).

Phenotypic characterization of X-99 and X-99a

Table 1 summarizes the biological phenotypes of X-99 and X-99a compared with the phenotypes of their H3N2 parental viruses [Sh/87 and Sh/87 (hy)]. The phenotypic characteristics of an escape mutant X-99I–, derived by passage of X-99 with normal horse serum (NHS) and of another, X-99aE, derived by passage of X-99a with the X-99a-specific MAb, A-9, are also shown. It is clear that the reassortants X-99 and X-99a have derived their pleiotropic serological and other characteristics from their respective Sh/87 and Sh/87 hy parents along with acquisition of their surface glycoproteins. Analysis of the escape mutants also suggests that the HAs of X-99 and X-99a differ in at least two sites as defined by reactions with NHS and the X-99a-specific MACBs, A-9 and A-56.
Table 2. Antigenic relatedness of selected H3N2 strains as defined by reciprocal HI tests with polyclonal rabbit antisera

<table>
<thead>
<tr>
<th></th>
<th>X-31</th>
<th>X-37a</th>
<th>X-91</th>
<th>X-97</th>
<th>X-99a</th>
<th>Sh/87</th>
<th>Sh/87 (hy)</th>
<th>(Bj/89)</th>
<th>X-101</th>
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<td>X-37a</td>
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<td>100</td>
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<tr>
<td>(Ln/86)</td>
<td>X-91</td>
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<td>100</td>
<td>50</td>
<td>79</td>
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<td>34</td>
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<td>100</td>
<td>31</td>
<td>24</td>
<td>–</td>
<td>–</td>
<td>100</td>
</tr>
</tbody>
</table>

* Strains from which HA and NA antigens were derived are in parentheses.
† Percentage antigenic relatedness as determined by fractional dilution HI (homologous relatedness is 100%).
‡ Reassortant virus, X-97, and antiserum to Sich/87 were used in this analysis.

i.e. the antigenic and inhibitor phenotype are not linked (co-varying).

Antigenic characterization of X-99 and X-99a viruses and antecedent and succeeding H3N2 variants

The same magnitude of antigenic difference between X-99 and X-99a (60%) is found for their respective wild-type antecedents Sh/87 and Sh/87 (hy) (Table 2). Although not completely identical, Sh/87 and X-99 and Sh/87 hy and X/99a were found to be related by 71% and 89%, respectively, by HI analysis. In plaque neutralization tests, X-99 and X-99a and Sh/98 and Sh/87 (hy) share only 25% identity.

Regarding the antigenic relatedness of recent H3N2 strains, summarized in Table 2, there is the significant difference between X-99a and all other strains, and similarity between X-99 and the Leningrad and Sichuan viruses isolated in 1986 and 1987. The scant relationship between both X-99 and X-99a to their antecedents of earlier decades, HK/68 and Eng/72 is apparent.

The two Sh/87 HA variants, X-99 and X-99a, differ almost as much from one another as does the first epidemiologically significant H3N2 drift variant (Eng/72) from the antecedent 1968 prototype virus (Fig. 1). There are also differences between the reassortants with respect to their reaction in serological (HI) tests using sera of patients recently infected with Sh/87-like influenza virus. Geometric mean serum antibody titres was fourfold higher when X-99 was the test antigen and antibody increases after infection averaged 6.5-fold with X-99, and 2.5-fold with X-99a virus (data not shown). Whether indicative of differences amongst the reassortants in antibody binding affinity in the test system, or reflective of preferential infection and/or antigenic stimulation by the X-99-like phenotype in man, these results constitute further evidence of significant HA-mediated differences in X-99 and X-99a. The differing efficiency of MDCK-adapted and egg-adapted variants in demonstrating human antibody response (Schild et al., 1983) also may be relevant to the present observations.

Comparative immunogenicity of X-99 and X-99a viruses in mice

We designed an experiment to test the relative efficacies of X-99 and X-99a vaccines in protection of mice from challenge infection by the uncloned Sh/87 virus from which both reassortants had been derived. Groups of five mice were injected intraperitoneally with one or two doses of u.v.-inactivated X-99 or X-99a virus, then infected intranasally with Sh/87 virus 28 or 41 days later, respectively. Just prior to infection, serum HI antibody was measured and 3 days after infection infective murine pulmonary-derived virus was assayed in chick embryos. The virus recovered from infected mice was identical in phenotype (i.e. X-99-like) to the original
infecting virus. The HI antibody responses to identical viruses or to the Sh/87 challenge virus did not differ significantly. When these mouse antisera were employed in cross-HI tests of X-99 and X-99a viruses, as had been done with rabbit and ferret antisera, they demonstrated complete viral antigenic relatedness (103%). Concordant with this finding, both vaccines were found to be equally protective against the dose of the parent Sh/87 virus used in challenges following either primary or secondary immunization.

### Molecular basis of the differences between X-99 and X-99a

The envelope proteins of X-99 and X-99a were identified by HI and NI tests as being H3 and N2. The M1 proteins of both viruses, and by inference RNA 7, were identified as derived from the PR8 virus through the use of PR8 M1-specific MAb in ELISA (Johansson et al., 1989). The remaining viral proteins were identified as derived from the PR8 virus by PAGE of viral proteins (Ritchey et al., 1977) (results not shown).

That the different reactivity of X-99 and X-99a in HI tests with polyclonal antisera was not ascribable to intrinsic differences in their N2 neuraminidases or to different ratios of the HA in their virions was demonstrated by the antigenic identity of their NAs in cross-NI tests and also by the preservation of typical X-99a-like reactivity in an H3N1 (PR8) reassortant derived from X-99a (data not shown). Therefore, attention turned to HA as the determinant of the pleiotropic differences in X-99 and X-99a.

Sequencing of HA-coding RNA 4 demonstrated non-synonymous (coding) changes reflected in the amino acid differences shown in Table 3. Viruses reactive with X-99a specific MAb (hy phenotype) differed from non-reactive X-99 (ly phenotype) viruses by having serine rather than isoleucine at amino acid 186 of HA1. X-99aE, an escape mutant from the neutralization of X-99a with MAb, did not, as expected, show the above substitution at position 186, but did differ from all other viruses by an aspartic acid for tyrosine substitution at amino acid 159 which, like 186, is located in the antigenic site B (see Discussion).

### Discussion

The emphasis in these studies is not upon the frequently reported minor antigenic variations detectable only with MAbs, but rather on the antigenic variation that is sufficiently extreme to be of potential epidemiological and immunological significance. The present results add to previous evidence of influenza virus strain heterogeneity (Kilbourne, 1987; de Jong et al., 1988; Robertson et al., 1991; Katz & Robertson, 1992) and confirm that significantly different antigenic variants identifiable with polyclonal serum as well as MAbs may coexist within a given viral strain. Selection of antigenic variants need not be immunological (Kilbourne, 1980; Erickson & Kilbourne, 1980; Dietzschold et al., 1983). Rather, antigenic change may be the consequence of selection for the hy characteristic (Both et al., 1983), the result of host adaptation (Schild et al., 1983), or may be entirely fortuitous. The recovery of ‘Czech/89-like’ or ‘Guangdong/89-like’ antigenically distinct variants from the same isolate has also been described (Johansson & Kilbourne, 1992).

Recent studies suggesting a role for the host cultivation system in the selection of HA antigenic variants (Wood et al., 1989; de Jong et al., 1988) are relevant to the suggestion that egg-grown viruses tend to have greater antigenic diversity (Robertson et al., 1991; Wang et al., 1989; Katz & Robertson, 1992) and to the identification of certain sites on the HA molecule that appear to characterize egg adaptation of the virus. One study, however, demonstrated the identity of sequences obtained from an egg isolate and its corresponding clinical specimen (Rajakumar et al., 1990). A mutation site identified by Katz et al. (1990) as a probable determinant of host specificity is position 186, the site that apparently is critical in the distinction of the X-99 and X-99a antigenic phenotypes. Both variants, however, have been cultivated only in the chick embryo host, in which differences in viral yield are demonstrable with both the wild-type (Sh/hy and Sh/ly) and reassortant (X-99 and X-99a) pairs. It is worth noting that serine at position 186 was usual for most egg-grown and two MDCK-grown clones studied by Katz et al. with PCR, yet in the present case, isoleucine at this position characterizes our egg-grown high yield phenotype viruses. Although Sh/hy, Sh/ly, X-99 and X-99a differ at other sites, these pairs have no substitution differences in common other than at position 186. With a panel of anti-H3 MAbs, amino acid 186 maps to the antigenic site B at the top of

### Table 3. Amino acid differences among Sh/87 HA mutants and reassortants

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Sh/hy</th>
<th>X-99a</th>
<th>X-99aE</th>
<th>Sh/ly</th>
<th>X-99</th>
<th>X-991</th>
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<tbody>
<tr>
<td>133</td>
<td>S</td>
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<tr>
<td>157</td>
<td>S</td>
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<td>S</td>
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<td>S</td>
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<td>186</td>
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<td>K</td>
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<td>N</td>
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<tr>
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<td>S</td>
<td>F</td>
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<td>T</td>
<td>T</td>
<td>N</td>
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</tbody>
</table>

* Deletion.
the HA molecule and at the interface of antigenic sites A and B. This site has been associated with frequent antigenic changes in nature (Underwood, 1984).

It is not clear whether or not the inhibitor phenotype differences are defined also by changes at position 186. Escape mutants X-99I- from X-99, and X-99aE from X-99a, selected respectively with NHS and MAb A56, demonstrated no reversion at site 186 coincident with their changes in phenotype. Rather, a deletion of amino acids 157 and 158 occurred with X-99I-, and a tyrosine to aspartic acid substitution occurred at position 159 with X-99aE. These amino acids also lie within the antigenic site B and might influence binding of either antibody or inhibitor at position 186.

The control of influenza is dependent upon a global (WHO) surveillance system that collects and categorizes new viral isolates with respect to their antigenic identity. At present antigenic characterization of new isolates is based principally on HI tests with serum obtained from ferrets 14 days after infection. However, the present experiments demonstrate a lack of concordance of antigenic analysis results when antisera from other species (rabbit and ferret) are compared with mouse antisera. Furthermore, whether or not the X-99 and X-99a reassortants differ by 65% (ferret), 60% (rabbit) or not at all (mice), vaccines from both viruses were equally effective in protecting mice against infection with the uncloned parental Sh/87 virus. This result suggests that minor variation among vaccine candidate strains can be tolerated, particularly because it is uncertain which variant is truly representative of the original human virus. In this connection, intraisolate antigenic heterogeneity has been shown to exist even in humans by sequencing viral HAs directly from clinical specimens (Katz et al., 1990) or from separate clones derived from a single specimen (Robertson et al., 1991; Katz & Robertson, 1992).

Prior studies have attempted to assess the significance of minor HA antigenic variation with respect to cross-protection in animal models. Comparisons among these studies and between these studies and our own are confounded by differences in viruses, host species and experimental design. Our finding that BALB/c mice cannot detect antigenic differences in X-99 and X-99a as measured either by HI antibody response or cross-protection are in accord with the studies in A/J mice by Rota et al. (1989) with vaccinia virus recombinants containing variant influenza B virus HAs. Katz et al. (1987) also demonstrated cross-protection of ferrets immunized by infection with antigenically distinguishable MDCK cell- and egg-grown variants. However, Wood et al. (1989), studying similar H1N1 host-adapted variants found a concordance of antibody response with cross-protection in guinea-pigs, ferrets and hamsters.

Although mapping of HA epitopes with MAb's in combination with RNA sequencing has produced valuable information on virus structure, antigenic variation and evolution, pragmatic consideration of the epidemiological significance of antigenic variation and vaccine choice must depend also on the use of polyclonal antibody for analysis of strain differences.

Ideally, whatever animal species is used for strain antigenic characterization, immunization should be carried out with non-replicating virus to forestall the selection of HA antigenic mutants best suited for replication in that particular host, but not necessarily representative of the input immunizing virus. Further research is needed on antigenic characterization as well as correlated studies on protection in humans, using viral replication quantification as the endpoint. Such experiments should aid in the perennial task of vaccine strain selection.

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References


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