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Human sera possess a limited antibody repertoire to influenza neuraminidase antigenic variants selected *in vitro*

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SUMMARY

Four antigenic variants of the neuraminidase (NA) of A/Texas/77 (H3N2) virus were selected using monoclonal antibody at a frequency of one variant in 10^5 parental virions. The antigenic variants failed to react serologically with the monoclonal antibody used for their selection *in vitro*. The antigenic variants failed also to react serologically with a proportion of sera from children and adults although all of the sera reacted with the parental A/Texas/77 virus. Thus, certain human sera have a restricted antibody repertoire to influenza NA antigen which might enable virus antigenic variants to avoid anti-NA antibody-mediated neutralization in nature.

INTRODUCTION

The neuraminidase (NA) is an integral membrane protein of influenza viruses composed of four identical polypeptide chains and possessing a complex antigenic structure with at least three non-overlapping antigenic areas (Webster *et al.* 1982*b*; Fields, Winter & Brownlee, 1981). Antibodies to NA inhibit elution of virus from infected cells (Seto & Rott, 1966) and reduce virus plaque size (Jahiel & Kilbourne, 1966) and are considered to contribute to protection or recovery from influenza in man. The neuraminidase, as well as the haemagglutinin (HA) undergoes antigenic drift (Paniker, 1968), the minor antigenic variation which results from spontaneous point mutation during viral replication followed by a selection of antigenic mutants in a partially immune host population (Webster *et al.* 1982*a*). *In vitro* selection of antigenic variants of the HA obtained by growing the virus in the presence of subneutralizing doses of monoclonal and polyclonal antibodies to haemagglutinin has proved useful in understanding the mechanism of antigenic drift. (Webster & Laver, 1975; Gerhard & Webster, 1978; Laver *et al.* 1979; Yewdell, Webster & Gerhard, 1979; Natali, Oxford & Schild, 1981). More recently, similar experiments have been performed to study this phenomenon using NA (Webster *et al.* 1982*b*). We have demonstrated previously that antigenic HA variants could be distinguished from the parental virus by a lower reactivity with

heterogeneous human sera (Natali *et al.* 1981, 1982). In the present study monoclonal antibody against viral neuraminidase of A/Texas/77 virus was used to select antigenic variants, which have been analysed in comparison with parental virus to determine if a restricted antibody repertoire also exists for this virus structural antigen. We demonstrate that certain human sera have a limited antibody repertoire and do not recognize virus NA with a change in a single epitope. Thus, together with the previous demonstration of restricted antibody repertoire to the HA antigen (Natali *et al.* 1981), an explanation is provided for the apparent paradox that newly arising antigenic variants would require changes in several epitopes to escape neutralizing atibody and spread in the community (Yewdell *et al.* 1979) but that the frequency of occurrence of such variants would be extremely low in theory and would approximate to 1 in 10^{24}.

MATERIALS AND METHODS

Viruses

Virus A/Texas/1/77 (H3N2) was grown in embryonated eggs and the uncloned infectious allantoic fluid was used as parental virus for the selection of antigenic variants.

Monoclonal anti-NA influenza antibodies

The procedures for producing the monoclonal anti-NA antibodies used for the selection of virus variants have been described in detail elsewhere (Koprowski, Gerhard & Croce, 1977; Webster *et al.* 1982*b*). The monoclonal antibody preparation used here was mouse ascitic fluid 123/1, kindly provided by Dr. R. G. Webster, with an elution inhibition (EI) titre (Appleyard & Oram, 1977) of $\geqslant$ 32000 and a neuraminidase inhibition (NI) titre of 3200.

Selection of virus antigenic variants

The selection of variants was carried out in the allantois on shell system (Yewdell *et al.* 1979; Natali *et al.* 1981) in absence or presence of hybridoma anti-NA antibodies. Dilutions of viruses were preincubated for one hour at room temperature in culture medium with or without undiluted monoclonal antibody. The antibody also inhibited the haemagglutinin activity with a haemagglutinization inhibition (HI) titre of 320 (see below). Egg pieces were then added to the virus–antibody mixtures and samples were incubated for another 30 min at room temperature. Following this absorption period the medium was removed and replaced by fresh virus-free medium with or without monoclonal antibodies at the same concentration as present during infection. This protocol was carried out to prevent any residual un-neutralized parental virus from infecting egg bits during the succeeding incubation period and to allow anti-NA antibody to be present to inhibit virus release (Kilbourne *et al.* 1968). After three days incubation at 37 °C, egg-piece cultures were assayed for the presence of virus by addition of 0·5% human RBC. Virus yields from the individual egg pieces were tested using NI and EI (Appleyard & Oram, 1977) with monoclonal antibodies used in the selection process.

Characterization of antigenic variants

The single radial haemolysis SRH (Schild, Pereira & Chakraverty, 1975; Oxford *et al.* 1981*a*) and NI (Aymard-Henry *et al.* 1973) tests used for the assay of antibody to HA and NA of new antigenic variants have been described previously.

Human sera

Unselected sera from children aged 1–5 years and adults aged 18–32 years were collected in Parma (Italy) during 1981–2.

SDS polyacrylamide gel electrophoresis

Chick embryo fibroblast cells (CEF) were infected with antigenic variants and parental virus and labelled with [^{35}S]methionine (10 μCi/ml) 8 h post-infection for 30 min. Samples were then prepared and used for PAGE as described elsewhere (Inglis *et al.* 1976; Oxford *et al.* 1981*b*). The polypeptides were analysed on gels with 12·5% polyacrylamide. To enhance detection of viral glycoproteins 4 M urea was added to the separation gel.

Analysis of NI antibody in human sera

Equal volumes of human sera and A/Texas/77 virus or its antigenic variants at appropriate dilutions (i.e. those giving an absorbance of 0·45–0·85 after 18 h incubation with fetuin) were mixed, and incubated at 37 °C for 1 h. Control samples consisted of viruses incubated with saline. Residual neuraminidase activities were determined by the method described by Aymard-Henry *et al.* (1973). Dilutions of sera which gave 50% inhibition of the neuraminidase activity of the control viruses were considered to contain antibody to NA.

RESULTS

Antigenic variants of A/Texas/77 (H3N2) virus were selected with monoclonal antibody to the viral neuraminidase and the freqency of occurrence of the variants was approximately 1 in 10^5 (data not presented). These antigenic variants were cloned in embryonated eggs in the presence of monoclonal antibody, and compared serologically to the parental virus in the EI test. No significant reactivity with the monoclonal antibody used in their selection was detected, whereas the parental virus reacted with a high EI titre ($\geqslant$ 32000). In addition, the NA variants showed differences in HI reactivity and they appeared to represent double antigenic variants (see below) of the parental virus (Table 1). However, the variants were still H3N2 subtype, as demonstrated by specific tests with anti-H3N2 chicken hyperimmune sera and post-infection ferret antiserum (data not presented).

Certain of the antigenic variants, designated HN1, HN2, HN3 and HN4, were then used in SRH and NI tests with human sera to determine if they could be distinguished antigenically from wild-type virus and to determine any differences in the serological reactivity of human sera to viral HA and NA of the parental virus and the antigenic variants (Tables 2 and 3). The tests performed confirmed the antigenic differences between parental virus and antigenic variants detected

Table 1. *Serological analysis of influenza virus antigenic variants selected* in vitro *using anti-NA monoclonal antibody*

Viruses	EI titre of 123/1	NI titre of 123/1	HI titre of 123/1
Parent A/Texas/77	32000	3200	320
Antigenic variants			
HN1	200	⩽ 20	⩽ 20
HN2	200	⩽ 20	⩽ 20
HN3	200	⩽ 20	⩽ 20
HN4	200	⩽ 20	⩽ 20

Dilutions of monoclonal antibody 123/1 were incubated with A/Texas/77 virus and the neutralized mixture titrated in egg pieces in the presence of excess monoclonal antibody. The four individual viruses (HN1–HN4) replicating in the presence of the antibody were selected for further study.

Table 2. *Frequency of occurrence of antibody to the neuraminidase of A/Texas/77 virus and antigenic variants in unselected human sera*

	Group	No. of sera tested	Percentage (number) of sera with NI antibody to virus antigenic variants			
			HN1	HN2	HN3	HN4
Sera with positive NI reactions with A/Texas/77 parent	Children	86	23·2 (20)	27·9 (24)	18·6 (16)	18·6 (16)
	Adult	126	25·3 (32)	30·1 (38)	39·6 (50)	23·0 (29)
Sera with no NI reaction with A/Texas/77 parent	Children	64	17·1 (11)	7·8 (5)	4·6 (3)	23·4 (15)
	Adult	24	25·9 (7)	54·1 (13)	41·6 (10)	37·5 (9)

Table 3. *Frequency of occurrence of antibody to the haemagglutinin of A/Texas/77 and antigenic variants in unselected human sera*

	Group	No. of sera tested	Percentage (number) of sera with SRH antibody to virus antigenic variants			
			HN1	HN2	HN3	HN4
Sera with positive SRH reactions with A/Texas/77 parent	Children	62	20·9 (13)	24·1 (15)	20·9 (13)	22·5 (14)
	Adult	85	18·8 (16)	12·9 (11)	16·4 (14)	14·1 (12)
Sera with no SRH reaction with A/Texas/77 parent	Children	88	4·5 (4)	6·8 (6)	9·0 (8)	4·5 (4)
	Adult	65	7·6 (5)	6·1 (4)	4·6 (3)	3·0 (2)

above using monoclonal antibody, and also showed a consistent difference between the serological reactivity of NA of parental virus and that of the antigenic variants with sera from both children and adults (Table 2). Thus, all four antigenic NA variants failed to react with a proportion of the sera although the sera all reacted with the NA of the parental A/Texas/77 virus. Variants HN3 and HN4 failed to react with 81·4% of individual children's sera and with 60·4% and 77·0% respectively of adult sera. A degree of serological variation was detected between the ability of the four antigenic variants to react with the children's or adult sera,

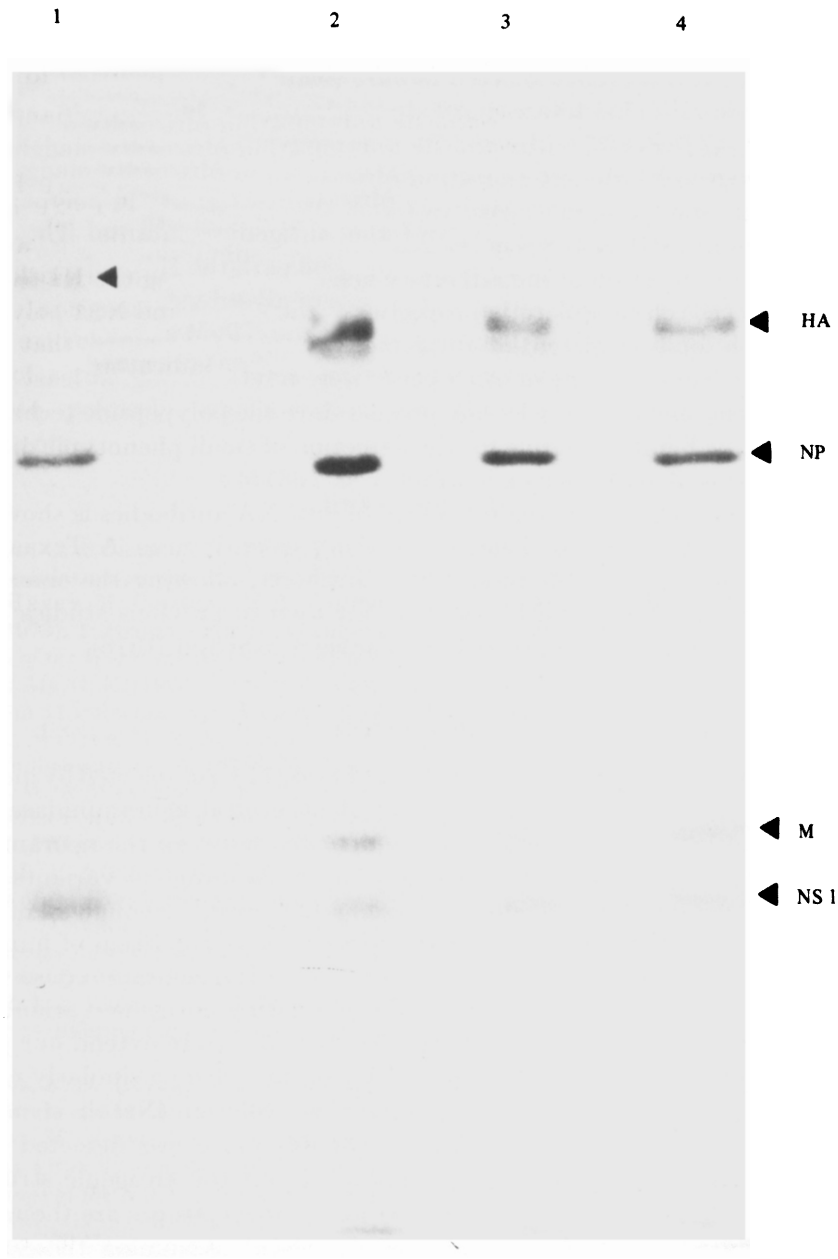

Fig. 1. Electrophoretic migration rates of viral structural and non-structural polypeptides of influenza A/Texas/77 and three NA variants derived from it. 1, A/Texas/77; 2, antigenic mutants HN 1; 3, antigenic mutants HN 2; 4, antigenic mutants HN 3. CEF cells were infected with viruses and 8 h post infection were pulsed with [^{35}S]methionine. Cell lysates were prepared and analysed using a 12·5 % polyacrylamide gel. Virus-induced polypeptides were identified in control experiments in which purified virus preparations were co-migrated with radio-labelled virus samples and by using the standard molecular weight markers (Oxford *et al.* 1981*b*). HA, haemagglutinin (note migration difference of parent and variants); NP nucleoprotein; M, matrix protein; NS1, non-structural protein no. 1; NA, is not resolved in these gels and probably co-migrates with NP polypeptide.

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although all the viruses were selected using the same monoclonal antibody. The explanation for these differences is not apparent, but a similar variation was noted previously with HA mutants selected *in vitro* (Natali *et al.* 1982).

The electrophoretic characteristics of viral polypeptides induced in chick embryo fibroblasts by A/Texas/77 virus and its antigenic variants were also compared. As shown in Fig. 1, a different migration rate of haemagglutinin polypeptide was noted between the parental virus and the antigenic mutants. These double antigenic variants, with changes in the HA as well as in the NA antigen, also failed to react with a proportion of human sera when analysed using the HI test (Table 3). In contrast, the electrophoretic properties of the NP, M and NS1 polypeptides were identical for the parental antigenic variants, indicating that random laboratory mutational events in other genes were not occurring, at least to a high frequency, during the *in vitro* selection process since the polypeptide technique has been shown to be highly sensitive for the detection of small phenotypic differences between influenza A or B viruses (Oxford *et al.* 1981*b*).

Clear evidence of the neutralizing ability of anti-NA antibodies is shown in the present report, since the replication of the parental virus A/Texas/77 was completely suppressed by the monoclonal antibody, allowing the emergence of antigenic variants. Polyclonal antisera to NA used in previous studies also had significant virus neutralizing properties (Haaheim & Schild, 1976).

DISCUSSION

Analysis of the four antigenic variants of A/Texas/77 virus selected by incubating the parent virus with monoclonal antibody to the viral neuraminidase showed clearly that antigenic differences could be detected between the neuraminidases of antigenic variants and parental virus. Moreover, the antigenic variants failed to react with a proportion of both children's and adult's sera, although all of the sera reacted with the parent virus. It would appear that a proportion of human sera possess a limited antibody repertoire to influenza virus neuraminidase which is restricted to a single epitope, thus potentially allowing antigenic variants of NA to escape virus neutralization in nature. The data therefore extend our previous studies with influenza A/Texas/77 virus HA variants where a similarly restricted antibody repertoire was noted, particularly in children (Natali *et al.* 1981). However, in the present study no significant difference was detected between children's and adult sera. Much less is known about the antigenic structure of influenza virus NA compared to HA but at least three epitopes are thought to be present on the NA molecule (Webster *et al.* 1982*a*). Assuming these are non-overlapping antigenic areas, the frequency of occurrence of antigenic variants to NA with changes in all three epitopes would be extremely low, and such variants would be unlikely to arise and spread in nature. Indeed, our studies indicate that changes in all three epitopes are probably not required to produce an antigenic variant of epidemiological significance. A mutational change in a single epitope, at least the epitope defined by monoclonal antibody 123/1, would appear to be sufficient to produce an antigenic variant which was not recognized by antibody in a large section of the population. The study could now usefully be extended to include monoclonal antibodies to other epitopes on the NA and to include antigenic

variants produced by sequential cultivation virus in the presence of different monoclonal antibodies.

It was of particular interest that 'double' mutants to both HA and NA were obtained in the present study, although we consider that the selection of HA mutants was fortuitous. Monoclonal antibodies to viral NA may cause steric effects, thereby apparently inhibiting HA at the same time (Webster *et al.* 1982*b*). Alternatively, but less likely, it is possible that non-specific inhibitors of HA in the monoclonal antibody preparation exerted a selective pressure on virus HA, thus resulting in mutants with both altered HA and NA.

Finally, antigenic variants of NA could be used to determine the precise number of amino acid changes in the NA (Blok & Air, 1980) needed to produce an antigenic variant of significance in terms of the epidemiology of the virus and also to investigate precisely which regions of the molecule constitute antigenic determinants.

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