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Frequency of naturally occurring antibody to influenza virus antigenic variants selected *in vitro* with monoclonal antibody

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SUMMARY

Antigenic variants of A/Texas/77 (H3N2) virus were selected *in vitro* using monoclonal antibody to virus haemagglutinin (HA). The antigenic variants and parental A/Texas/77 viruses were used to evaluate the frequency of anti-HA antibodies in the sera of children and adults using single-radial-haemolysis (SRH) tests. Twenty to 41% of selected sera from adults, which contained antibody to the parental A/Texas/77 virus, failed to react with the different antigenic mutant viruses. A higher proportion of sera from children (37–58%) failed to react with the antigenic variants. Certain human sera and particularly those of children would appear to possess a more limited antibody repertoire to influenza HA, potentially allowing new antigenic variants to escape neutralization and spread in the community.

INTRODUCTION

Monoclonal antibodies have been used for investigations of antigenic and corresponding structural variation in the HA of influenza A virus strains (Koprowski, Gerhard & Croce, 1977; Gerhard & Webster, 1978; Laver *et al.* 1979; Yewdell, Webster & Gerhard, 1979). However, the relevance of the antigenic determinants delineated by monoclonal antibodies to the epidemiology and immunology of influenza in man has not been established to date. The object of the present study was to determine whether antigenically variant influenza A viruses selected *in vitro* using monoclonal antibodies to HA were changed, compared with the parental virus, in their ability to react with human sera. Such differences in serological reactions are demonstrated here and suggest that the monoclones studied were directed towards immunological determinants of the HA recognized by man and included in the 'repertoire' of the antibody responses to HA. In particular, children's sera possessed a more limited anti-influenza virus antibody repertoire which allows the hypothesis that novel antigenic mutants could be selected in nature and spread in young children.

MATERIALS AND METHODS

Viruses

Influenza A/Texas/77 (H3N2) virus was grown in embryonated eggs by standard techniques and the allantoic fluids were used as parental virus for the selection of antigenic variants.

Monoclonal anti-influenza HA antibodies

The procedures for producing the monoclonal anti-HA antibodies used for the selection of virus variants have been described in detail elsewhere (Koprowski *et al.* 1977). The monoclonal antibody preparation used here was mouse ascitic fluid designated 171/7 and kindly provided by Dr R. G. Webster.

Selection of virus antigenic variants

The selection of variants was carried out in the allantois-on-shell system (Yewdell *et al.* 1979) in the presence of hybridoma anti-HA antibody. Briefly, dilutions of viruses were pre-incubated for 1 h at room temperature in culture medium with or without monoclonal antibody diluted 1 in 2000. Egg pieces were then added and samples were incubated for another 30 min at room temperature. Following this infection 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 procedure reduced the possibility of residual unneutralized parental virus infecting the egg pieces during the following incubation period. After 3 days incubation at 37 °C, egg piece cultures were assayed by addition of 0·5 % RBC and virus yields from the individual egg pieces were tested in HI assays with the monoclonal antibodies used in the selection process.

Characterization of antigenic variants

The single-radial-haemolysis (SRH) test was used for the assay of antibody to the HA of new antigenic variants, as described previously (Schild, Pereira & Chakraverty, 1975; Oxford *et al.* 1981)

Human Sera

Sera from children aged 1–5 years and adults aged 18–32 years were collected in London during 1979–80.

RESULTS

Monoclonal antibody to influenza A/Texas/77 (H3N2) virus was used to select antigenic variants from parental virus stocks. The frequency of antigenic variants thus selected and which failed to react in HI test (titres $\leqslant 500$) with monoclonal antibody was $10^{-5\cdot3}$. The antigenic mutants were repassaged at terminal dilution in the presence of a 1 in 2000 dilution of the monoclonal antibody and the virus harvests used to infect eggs to prepare a working stock of virus. Since passage of influenza A viruses in the absence of antibody pressure can lead on occasion to the selection of antigenic variants (Kilbourne, 1978), control virus clones were obtained from the parental A/Texas/77 virus using normal ascitic fluids and the

Table 1. *Serological analysis of influenza antigenic mutant viruses selected by anti-HA monoclonal antibody*

Viruses	HI titres with following sera: Anti-HA monoclone 171/7	Post infection ferret serum† to A/Vic/76 (H3N2)	Representative human antiserum
Parent A/Texas/77 virus	8000*	160	40
Antigenic variants 2	< 500	20	< 10
7	< 500	20	< 10
18	< 500	160	< 10
21	< 500	< 20	< 10
28	< 500	< 20	NT
29	< 500	< 20	NT

An additional 8 viruses were cloned separately in the presence of control mouse ascitic fluid. These viruses could not be distinguished from the parental A/Texas/77 virus when tested against 17 children's sera, monoclone 171/7 or the post infection ferret sera.

† This was the single ferret serum from 18 tested which discriminated serologically between the antigenic mutants and the parent A/Texas/77 virus. (Oxford, unpublished data).

NT not tested.

above procedures. The antigenic mutants and control viruses were analysed using HI or SRH tests with the 171/7 monoclonal antibody and with post-infection ferret and human sera to A/Texas/77 (Table 1). The six antigenic mutants analysed in the present study all failed to react with anti-HA monoclone at a dilution of 1/500, whereas the A/Texas/77 parent virus reacted at a dilution of 1 in 8000. Most of the antigenic mutants, with the exception of mutant 18, also failed to react in HI and SRH tests with a selected post-infection ferret serum. The antigenic mutants tested also failed to react with a selected post-infection children's serum. In contrast, eight viruses selected under identical conditions but using a control ascitic fluid reacted equally as well as the parent A/Texas/77 virus with the anti HA-monoclone 171/7, the post-infection ferret serum and 17 post-infection sera from children.

Sero-epidemiological analysis using antigenic variants

The six antigenic variants of A/Texas/77 described above were incorporated into the agarose of SRH plates to determine if they could be distinguished antigenically from the parental virus using human sera (Plate 1). All antigenic variants failed to react with a proportion of children's sera, although the same children's sera reacted with the parent A/Texas/77 virus. Thus, variant 2 failed to react with 17 of 38 sera (55%) of individual children compared to the parental virus A/Texas/77, which reacted with all of the sera (Table 2). Variant 2 also showed reduced serological reactivity with adult sera and failed to react with 20% of the sera, although the same sera reacted with the parent A/Texas/77 virus. A degree of quantitative variation was noted between the ability of the different antigenic variants to react with children's sera, although all the viruses had been selected using the same monoclonal antibody. Thus, mutant No. 18 failed to react with 58% of the sera which reacted with the parental virus compared to variant

Table 2. *Frequency of antibody to A/Texas/77 virus and to antigenic mutants*

		No of sera tested	Percentage of sera with SRH antibody to virus mutant					
			2*	7	18*	21	28	29
Sera with positive SRH reactions with A/Texas/77 parent	Children	38	45	55	42	63	61	53
	Adult	41	80	73	76	76	63	59
Sera with no SRH reactions with A/Texas/77 parent	Children	172	4	0	0·6	4	3·5	0·6
	Adult	156	0	0	0	0	0	0·6

* Significant difference in reactivity between children's and adult sera ($P < 0{\cdot}01$)

No. 21 which failed to react with 37% of the sera. The reason for this variation is not known but Laver *et al.* (1980) also noted that certain virus variants selected with a single monoclone differed antigenically within a group.

A consistent difference was noted between the ability of the childrens and the adult sera to react serologically with the antigenic variants. Forty two–63% of children's sera and 59–80% of adult sera reacted with the different antigenic mutants, whereas all the sera reacted with the parental virus. The quantitative antibody response, excluding analysis of negatively reacting sera, to the parental and to the antigenic variants was very similar in sera from both children and adults. Thus, for children's sera reacting in the SRH plates with parental virus and antigenic variant No. 2 a mean zone diameter of 22·2 and $22{\cdot}7 \pm 5{\cdot}6$ mm^2 respectively was detected. A low proportion of children's sera possessed antibodies reacting with certain of the antigenic variants, but had no detectable antibodies reacting with the A/Texas/77 parent virus. Thus, approximately 4% and 0·6% of children's sera, which did not react with the parent A/Texas/77 virus, reacted with variant Nos 2 and 18 respectively.

DISCUSSION

Previous studies suggested that as many as four different antigenic sites could be located on the HA of influenza A virus, although these might overlap to some extent (Yewdell *et al.* 1979). Antigenic variants selected with a single monoclonal antibody to HA occurred with a frequency of approximately 1 in 100000 in previous studies and also in the present report. The frequency of occurrence of antigenic variants with changes in all four independent sites would be extremely low in theory and would approximate to 1 in 10^{24} (Yewdell *et al.* 1979). In previous studies polyclonal animal sera neutralized the antigenic variants to the same extent as parental virus suggesting that new antigenic variants would be easily neutralized in nature (Laver *et al.* 1979; Yewdell *et al.* 1979). In particular, since an immune individual might be expected to produce antibodies against all four

of the antigenic sites on the HA molecule it has been particularly difficult previously to understand how such variants could arise in nature at such low frequencies and subsequently could have escaped neutralization and spread in the community (Laver *et al.* 1979; Yewdell *et al.* 1979).

In the present paper we report that a proportion of the human antisera is able to distinguish between antigenic variants, selected *in vitro* after a passage in a single monoclonal antibody preparation and presumably therefore having an antigenic change in a single epitope, and the parental virus. It would appear that compared to certain experimental animals (Yewdell *et al.* 1979), a proportion of human sera, and particularly children's sera, possess a more limited anti-influenza virus antibody repertoire, which is restricted to one or perhaps two non-overlapping epitopes, thus potentially allowing such antigenic variants to escape neutralization and spread in the community. Changes in all four antigenic sites would seem not to be a prerequisite for the selection of a significant variant of epidemiological significance. We can hypothesize therefore that comparable novel antigenic virus mutants could be selected in nature and spread, particularly in young children. Children frequently experience the highest attack rates during influenza epidemics and may be a primary source of virus spread in the home and community (reviewed by Kilbourne, 1975; Dowdle *et al.* 1980). Additional data to substantiate this hypothesis is provided by Haaheim (1980) who analysed sera using the HI test and by a comparative analysis of anti-HA antibody in young children and adults which indicated that young children produce antibody which is highly specific for the HA of the infecting influenza A virus and which does not cross react with other influenza variants of the same antigenic subtype (Oxford *et al.* 1981).

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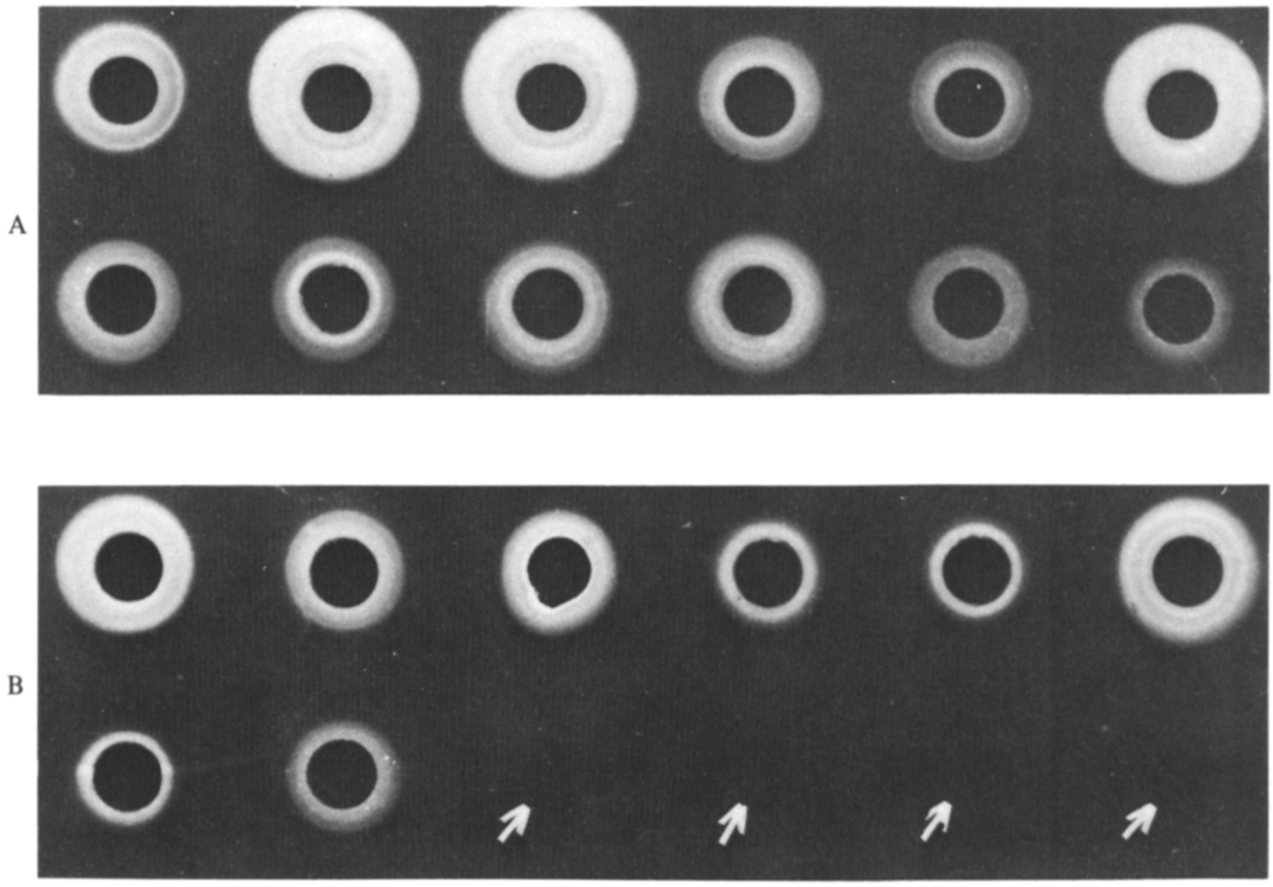
EXPLANATION OF PLATE

PLATE 1

Comparative serological reactivity of children's sera with A/Texas/77 parental virus and antigenic variant 18. 10000 HA units of virus were mixed with 1 ml of 10% sheep erythrocytes in 0·05 M Hepes buffer pH 6·5. Chromium chloride diluted in 1 in 400 from a 2·25 M solution was added for 5 min at 4 °C. After several washes in PBS, virus-sensitised red blood cells (0·25 ml) and fresh guinea pig complement (0·15 ml) were added to 2·5 ml agarose gel. Ten μl volumes of the 12 heat inactivated human sera were added to the wells in the gel immunoplate and the plates incubated over-night at 37 °C. Zone sizes of haemolysis were measured using a calibrating viewer. A A/Texas/77 (H3N2) virus incorporated in the immunoplate. B antigenic variant No 18 incorporated in the immunoplate. Note the absence of reactivity of 4 antisera (arrowed) in the SRH plate containing the antigenic mutant compared to the positive reaction of the same sera in the parent A/Texas/77 virus SRH plate. Other sera show either the same quantitative reaction in both plates or, in a low proportion of sera, a reduced reaction in the SRH plate with the antigenic variant.



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