Effect of Test System on the Ability of Monoclonal Antibodies to Detect Antigenic Drift in Influenza A (H1N1) Virus Haemagglutinins

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SUMMARY

Results of analysing antigenic variation in the haemagglutinin (HA) molecule of naturally occurring influenza A (H1N1) viruses from 1977 to 1979 with monoclonal antibodies were found to be dependent in some instances on the test system used. In several instances A/USSR/90/77 HA-specific monoclonal antibodies had sharply reduced haemagglutination-inhibition (HI) titres with variant virus although they bound to the variant and A/USSR/90/77 HAs with similar efficiencies as judged by titration in a sensitive and accurate solid-phase immunofluorimetric assay. In another instance, the converse situation was observed: monoclonal antibodies having a reduced efficiency of binding to the HA of a variant virus nevertheless had comparable HI titres with the variant and with A/USSR/90/77. The chemical basis and epidemiological significance of these observations remain to be elucidated. Nevertheless, the finding that the reaction of monoclonal antibodies can, in some cases, be markedly dependent on the test system employed is of significance for the efficient design and correct interpretation of immunochemical studies which employ monoclonal antibodies to investigate the basis for variation in influenza strains.

INTRODUCTION

The genetic and chemical basis for variation in the antigenic specificity of influenza virus haemagglutinins (HAs) needs to be determined so as to understand the factors which lead to the appearance of new epidemic strains, and to improve vaccines used for their control. As one approach to this objective we have been observing the variation in influenza A (H1N1) viruses using monoclonal antibodies to the HA of A/USSR/90/77 (Webster et al., 1979). The A/USSR/90/77 strain is representative of the H1N1 virus that reappeared as a human epidemic strain in 1977, following which time several antigenic variants were detected (Kendal et al., 1979). By a prospective study with monoclonal antibodies of variation in naturally occurring H1N1 viruses since 1977 we hope to learn whether change at any particular epitope(s) might be associated with survival of H1N1 viruses, after the originally susceptible population develops immunity.

Analysis of antigenic determinants using monoclonal antibodies was first done by the haemagglutination-inhibition (HI) test because of its simplicity and because of the general assumption that antibodies which inhibit haemagglutination effectively protect against influenza virus infection (Virelizier, 1975; Schulman, 1975). However, immunoprecipitation reactions with heterogeneous animal sera (Schild, 1970), as well as analysis of monoclonal antibodies by immunoassay (Gerhard et al., 1980), indicate that some antibodies may bind to HA that are not detected by HI. Furthermore, a study has been described which shows that
the total serum antibody responses detected by an immunometric assay do not always correspond with antibody responses measured by HI (Six & Kasel, 1978). Therefore, the present investigation was undertaken to compare binding of selected HA-specific monoclonal antibodies to influenza A (H1N1) variants by HI and by an immunometric assay to provide more complete information about the nature of antigenic variation in naturally occurring influenza A (H1N1) strains.

METHODS

Influenza viruses. Variants were selected from naturally occurring virus isolates to include examples that had unique reaction patterns with the available monoclonal antibodies in HI. A/England/1/51 was included because it was known to have low HI reactivity with two monoclonal antibodies (22/1 and 110/1) that inhibited all recently isolated influenza A (H1N1) virus variants (Webster et al., 1979), and because we wanted to ensure that our tests included examples of low HI reactivity with each monoclonal antibody. Each virus was propagated by inoculating the allantoic cavity of 10-day-old embryonated chicken eggs with approx. 10^4 egg infectious doses, and after incubation for 2 days at 34 °C the harvested allantoic fluids had HA titres ranging from 256 to 1024.

Haemagglutination and HI tests. These were done by the microtitre method with chicken red blood cells (Dowdle et al., 1979). Ascitic fluids were treated with receptor destroying enzyme, to inactivate non-specific inhibitors. Antibody-containing specimens were serially diluted in bulk volumes for each day's test so that aliquots of identical dilutions were added by dropper to different virus antigens in comparative tests, which always included each variant virus. This procedure was done to increase reproducibility compared to the use of microtitre loops in the HI tests. Titre differences of ≥ fourfold were considered significant.

Ascitic fluid. Ascitic fluids containing monoclonal antibodies specific for the HA of A/USSR/90/77 (H1N1) viruses were prepared as previously described using the P3/X-63-Ag8 ∂1k1 secretory cell line (Webster et al., 1979). Heavy-chain specificities were determined by double-immunodiffusion tests with hybridoma tissue culture fluids and specific heavy-chain antisera (Litton Bionetics, Kinsington, Md., U.S.A.), and equilibrium dissociation constants were determined as described by Fazekas de St. Groth (1961).

Evidence that each preparation was monospecific was obtained as follows: firstly, by demonstrating that each ascitic fluid reacted in HI tests with A/USSR/90/77 virus, but did not react in neuraminidase-inhibition assays with A/USSR/90/77 virus or in immunoassays that can detect reactions with type-specific or host antigens (Van Wyke et al., 1980; Phillips et al., 1980a); secondly, by determining that each IgG-class preparation selected mutants from cloned A/USSR/90/77 virus at a frequency of about 10^{-4} to 10^{-5} (Nakajima & Kendal, 1981), when it is known that mutants cannot be selected at detectable frequencies (< about 10^{-8}) when cloned virus is grown in the presence of two monoclonal antibodies having specificities for different loci on influenza HA (Yewdell et al., 1979; Webster & Laver, 1980); and thirdly, by showing that all influenza-specific antibody in the preparation 22/1 sedimented at about 18S in sucrose gradients and was of IgM class when determined by indirect immunofluorimetric assay (IIFA) using class-specific conjugates proven to be monospecific for IgM or IgG mouse antibodies (N. Gonchoroff & A. P. Kendal, unpublished results). It is quite improbable that a mixture of hybridoma cells would both be secreting influenza-specific antibody of IgM class.

Immunometric assay. Binding of mouse monoclonal antibodies to influenza viruses was measured by a solid-phase IIFA procedure illustrated schematically in Fig. 1 and previously described in detail (Phillips et al., 1980a). Briefly, the IgG fraction of serum collected from a rabbit which had been hyperimmunized with purified A/Brazil/11/78 (H1N1) virus was covalently coupled to 1-1 μm diam. polyaminostyrene (PAS) beads by a diazotization
Monoclonal antibody reactions with influenza virus

Polyaminostyrene (PAS) beads

Hyperimmune antiviral rabbit IgG

Diazotization

IgG-coated PAS beads

Mouse monoclonal antibody

Solid-phase beads with virion and subvirion antigens

Immunological coupling

Infected fluid containing virions, subvirion antigens and host materials

Reactivity with test sample

Indirect labelling

Solid-phase virus antigen–monoclonal antibody complex

FITC-conjugated goat anti-mouse Ig

Fig. 1. Schematic representation of the solid-phase indirect immunofluorimetric assay for measuring the reaction of mouse monoclonal antibodies with viral antigen.

reaction (Reimer et al., 1978). About 3.7 x 10^7 of the PAS/IgG beads were dispensed into 12 x 75 mm glass tubes and 0.1 ml of virus-containing allantoic fluid with an HA titre of 256 added, this amount of virus corresponding to about 50% of the antigen-binding capacity of the beads. After 1 h incubation at 37 °C 0.1 ml of each dilution of the mouse ascitic fluids was added and the mixtures were incubated for a further 1 h at 37 °C. The solid phase was then washed twice by centrifugation at about 2000 g for 15 min and resuspension in 2 ml phosphate-buffered saline. Mouse antibody bound to the solid phase was detected by addition of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig at a dilution capable of saturating antigen beads that bound maximum levels of mouse antibody. The final FITC-labelled complexes were washed three times by centrifugation and resuspension, and the amount of bound FITC quantified in a filter fluorimeter (Phillips et al., 1980b).

RESULTS

Reproducibility of IIFA

As previously reported (Phillips et al., 1980a) results of 30 repetitive determinations of background levels of fluorescence over several days established that relative fluorescent intensities of twice the background level corresponded to 15 deviations above the mean background reading. Analysis of the reactions of monoclonal antibody in the test showed that
Table 1. Reproducibility of indirect immunofluorometric assay for measurement of a mouse monoclonal antibody with influenza virus antigen immunologically coupled to solid-phase latex beads

<table>
<thead>
<tr>
<th>Monoclonal antibody dilution</th>
<th>Antigen</th>
<th>Relative fluorescence intensity</th>
<th>Coefficient of variation (C.V.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Overall mean</td>
<td>Range of daily means</td>
</tr>
<tr>
<td>1000</td>
<td>Virus</td>
<td>0.085</td>
<td>0.069-0.098</td>
</tr>
<tr>
<td>1000</td>
<td>Control</td>
<td>0.016</td>
<td>0.015-0.018</td>
</tr>
<tr>
<td>10000</td>
<td>Virus</td>
<td>0.042</td>
<td>0.039-0.045</td>
</tr>
<tr>
<td>10000</td>
<td>Control</td>
<td>0.015</td>
<td>0.015-0.016</td>
</tr>
<tr>
<td>20000</td>
<td>Virus</td>
<td>0.029</td>
<td>0.028-0.031</td>
</tr>
<tr>
<td>20000</td>
<td>Control</td>
<td>0.016</td>
<td>0.014-0.017</td>
</tr>
<tr>
<td>40000</td>
<td>Virus</td>
<td>0.021</td>
<td>0.020-0.021</td>
</tr>
<tr>
<td>40000</td>
<td>Control</td>
<td>0.015</td>
<td>0.014-0.016</td>
</tr>
<tr>
<td>10000000</td>
<td>Virus</td>
<td>0.016</td>
<td>0.015-0.017</td>
</tr>
<tr>
<td>10000000</td>
<td>Control</td>
<td>0.015</td>
<td>0.014-0.017</td>
</tr>
</tbody>
</table>

* Each dilution of monoclonal antibody was tested eight times on each of 4 days using as antigen both virus-infected allantoic fluid and non-infected allantoic fluid as control.

Table 2. Comparison of haemagglutination-inhibition (HI) test and indirect immunofluorometric assay (IIFA) for the detection of monoclonal antibodies to influenza A/USSR/90/77 (H1N1) haemagglutinin and antigenic variants of influenza A(H1N1) virus

<table>
<thead>
<tr>
<th>Antigen</th>
<th>W18/1</th>
<th>22/1</th>
<th>70/1</th>
<th>110/1</th>
<th>264/7</th>
<th>385/1</th>
</tr>
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<tbody>
<tr>
<td>A/England/1/51</td>
<td>HI</td>
<td>HI</td>
<td>HI</td>
<td>HI</td>
<td>HI</td>
<td>HI</td>
</tr>
<tr>
<td>A/USSR/90/77</td>
<td>3200</td>
<td>6900</td>
<td>950</td>
<td>950</td>
<td>600</td>
<td>130</td>
</tr>
<tr>
<td>A/Brazil/11/78</td>
<td>1600</td>
<td>6500</td>
<td>16000</td>
<td>20000</td>
<td>25600</td>
<td>29000</td>
</tr>
<tr>
<td>A/Lackland/3/78</td>
<td>1000</td>
<td>16000</td>
<td>9000</td>
<td>57000</td>
<td>1600</td>
<td>1000</td>
</tr>
<tr>
<td>A/Lackland/7/78</td>
<td>1600</td>
<td>6000</td>
<td>13200</td>
<td>26000</td>
<td>4000</td>
<td>44000</td>
</tr>
<tr>
<td>A/California/45/79</td>
<td>100</td>
<td>16000</td>
<td>22000</td>
<td>38000</td>
<td>25600</td>
<td>48000</td>
</tr>
<tr>
<td>A/USSR/90/79</td>
<td>1200</td>
<td>2200</td>
<td>14400</td>
<td>23000</td>
<td>25600</td>
<td>48000</td>
</tr>
<tr>
<td>A/Kumamoto/35/79</td>
<td>300</td>
<td>200</td>
<td>16000</td>
<td>14000</td>
<td>25600</td>
<td>20000</td>
</tr>
</tbody>
</table>

* Results, the mean of duplicate tests, are the reciprocal of the highest twofold dilution of ascitic fluid causing complete inhibition of 4 haemagglutinating units of virus.

† Results are the reciprocal of the highest dilution of ascitic fluid producing a relative fluorescent intensity of twice background interpolated from the mean of two or more values obtained with each of a series of 3.2-fold dilutions of ascitic fluid. Background, determined using uninfected allantoic fluid in place of virus-infected fluid, was 0.015 units of relative fluorescence intensity.

‡ Instances where a virus showed a difference from A/USSR/90/77 in only one test system (HI or IIFA) are underlined.

Positive reactions could be determined with good precision even at high antibody dilutions (Table 1). For this comparative study antibody titres were therefore interpolated as the reciprocal of the antibody dilution producing a fluorescent intensity of twice the background. Further analysis of reproducibility for measuring antibody titres was done by performing replicate titrations of one monoclonal antibody (22/1) at several fourfold dilutions with A/USSR/90/77 antigen eight times on each of 3 successive days. A mean titre of 26 500 was obtained, with the range of titre being 20 000 to 32 500 and an overall standard deviation of ±11% of the mean determination. The mean titre of this antibody preparation tested with A/USSR/90/77 antigen on four other occasions over a 5 month period was 23 000, with a standard deviation of ±22%. For monoclonal antibody 385/1 the standard deviation in three titrations with A/USSR/90/77 was ±13%. Results of testing monoclonal antibodies with heterologous viruses would be expected to incur additional errors, due to the effects of variations in antigen concentration or dissociation of antigen-antibody complexes at high dilutions of mouse monoclonal antibody. Inspection of results (Table 2) obtained with
Monoclonal antibody reactions with influenza virus

Fig. 2. Titration in the solid-phase indirect immunofluorimetric assay of individual mouse monoclonal antibodies specific for HA of A/USSR/90/77 (H1N1) with homologous and heterologous influenza A (H1N1) viruses. Solid-phase antigens were prepared by binding virus present in allantoic fluid infected with A/USSR/90/77 (O), A/England/1/51 (●) and A/Brazil/11/78 (▲) to polyaminostyrene beads coupled to IgG from serum of rabbits hyperimmunized with A/Brazil/11/78. Monoclonal antibodies were (a) 22/1 and (b) 264/7.

Reactions of different HA-specific mouse monoclonal antibodies with naturally occurring antigenic variants of human influenza A (H1N1) virus

Six monoclonal antibody preparations, shown by HI to have specificities for different epitopes of H1 haemagglutinin, were titrated by IIFA with several naturally occurring antigenic variants of influenza A (H1N1) virus. For this study, solid-phase antigens were prepared by adding allantoic fluids infected with the different influenza A (H1N1) variants to the beads with covalently bound IgG from the serum of a rabbit hyperimmunized with A/Brazil/11/78 (H1N1). The rabbit serum reacted in HI with all the tested variants to a titre within fourfold of that obtained with A/Brazil/11/78, although many of the viruses can be distinguished from each other by using a set of more highly specific convalescent-phase sera from infected ferrets (Kendal et al., 1979) and, as shown below, each selected virus has a unique reaction pattern with mouse monoclonal antibodies. In the IIFA, at least one monoclonal antibody preparation reacted with each variant virus to a titre within twofold of that obtained with the homologous A/USSR/90/77 antigen (Table 2). This confirmed that IgG from the rabbit hyperimmunized with A/Brazil/11/78 and coupled to the beads had a sufficiently broad specificity to bind the variant influenza A (H1N1) viruses in approximately equivalent amounts when solid-phase antigen was prepared. Therefore, loss of IIFA reactivity by specific monoclonal antibodies with different variant influenza A (H1N1) viruses (Fig. 2,
Table 2) was not an artefact of preparation of the solid-phase antigens using the variant viruses.

Comparison of HI and IIFA results showed that several discordant results were found in the detection of antigenic variation by the two methods (Table 2), and particular instances were observed of monoclonal antibody preparations that reacted quite well with viruses as judged by titration in the immunometric assay, but had poor HI activity. Examples of this are monoclonal antibody preparation 22/1 with A/England/1/51 virus, preparation 70/1 with A/Lackland/7/78 virus and preparation 385/1 with viruses A/England/1/51, A/California/45/78 and A/USSR/50/70.

In several of these cases residual HI activity was detected, but at a titre from 8- to 16-fold lower than the homologous reaction with A/USSR/90/77 (which is highly significant), whereas IIFA titres were at most threefold lower than homologous titres and would not normally be considered to provide evidence for changed epitopes in these cases. The observations were highly reproducible in repeated testing. One possibility for discordant HI and IIFA was that viruses were mixtures containing some HA recognized by the antibody (so that binding would be detected by IIFA) but a proportion of the HA molecules lacked the epitope recognized by the antibody (so that virus would be inhibited in HI). This was examined by cloning A/USSR/50/79 through a plaque-purification procedure, and retesting it. The plaque-purified virus, however, exhibited a similar reaction pattern as the starting material (results not shown). A further possibility was that the monoclones reactive by IIFA but not by HI contained not only antibody directed at HA, but also antibody specific for some other component present in the solid-phase IIFA antigen. This was unlikely to be the case for monoclon 70/1 which failed to react with A/England/I/51 virus in IIFA. Antibody preparations 22/1 and 385/1 were shown to be non-reactive in the IIFA with type-specific antigens of influenza A (H3N2) virus and did not react with H1N1 virus in neuraminidase-inhibition tests. Furthermore, virus non-reactive with preparation 385/1 in IIFA has been identified among laboratory-derived variants of A/USSR/90/77 (S. Nakajima & A. P. Kendal, unpublished results). Therefore, the observation of monoclonal antibody having reduced HI activity without corresponding reduction in IIFA titre was not an artefact of heterogeneity in virus antigen or monoclonal antibody.

Another discrepancy in HI and IIFA results was the finding that preparation 264/7 strongly inhibited the haemagglutinating activity of A/England/1/51 virus, although relatively low efficiency of antigen binding was detected in the IIFA, compared to the homologous reaction with A/USSR/90/77 (Table 2, Fig. 2b). A similar, but less pronounced, observation was made for monoclonal antibody preparation W18/1 with virus A/USSR/50/79. In other instances, however, IIFA and HI detected similar patterns of antigenic change in variant virus HAs away from A/USSR/90/77 as seen for monoclonal antibody preparation 70/1 and 110/1 with virus A/England/1/51; and for preparation 264/7 with viruses A/Brazil/11/78, A/Lackland/3/78, A/Lackland/7/78, A/California/45/78, A/USSR/50/79 and A/Kumamoto/35/79.

**Immunoglobulin class and subclass, and affinity of the hybridoma antibodies to A/USSR/90/77 (H1N1) HA**

The immunoglobulin class or subclass and equilibrium binding constant of the hybridoma antibodies were determined (Table 3) to establish whether there was any correlation between these properties and their ability to react in HI and IIFA. Since the P3/X-63-Ag8 line of myeloma cells used in preparation of the hybridoma cells secrete γ1, heavy chain (H), and κ1, light chain (L), various combinations of H and L chains would be possible in the Ig chains responsible for antiviral activity. IIFA and HI titre ratios with homologous A/USSR/90/77 antigen ranged from about 1:1 (antibody 22/1 and 110/1) to 8:1 (264/7), but there was no
Monoclonal antibody reactions with influenza virus

Table 3. Immunoglobulin isotype and affinity of hybridoma antibodies to the haemagglutinin molecule of A/USSR/90/77 (H1N1)

<table>
<thead>
<tr>
<th>Hybridoma antibody number</th>
<th>Heavy-chain isotype</th>
<th>Affinity* (κ × 10&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W18/1</td>
<td>y1</td>
<td>2.29 ± 0.16</td>
</tr>
<tr>
<td>22/1</td>
<td>y1, μ</td>
<td>ND†</td>
</tr>
<tr>
<td>70/1</td>
<td>y1</td>
<td>2.85 ± 0.61</td>
</tr>
<tr>
<td>110/1</td>
<td>y1, γ3</td>
<td>34.4 ± 2.1</td>
</tr>
<tr>
<td>264/7</td>
<td>y1, γ2a</td>
<td>77.9 ± 7.6</td>
</tr>
<tr>
<td>385/1</td>
<td>y1</td>
<td>49.6 ± 4.6</td>
</tr>
</tbody>
</table>

* Equilibrium binding constants were determined by equilibrium filtration; the larger the value of κ, the lower the affinity of the antibody.

ND, Not done because the γM (μ) antibody bound non-specifically to the cellulose filters.

consistent correlation between antibody class or subclass and relative ability to react in HI and IIFA. Firm conclusions cannot be drawn about the effect of heavy-chain composition on the intrinsic ability of each hybridoma antibody to detect antigenic variation because the antigens used in this study were pre-selected on the basis of known results to include variants with a variety of different reaction patterns.

Of five IgG class hybridoma antibody preparations tested with the homologous A/USSR/90/77 antigen, two (W18/1 and 70/1) were of relatively high avidity, having equilibrium dissociation constants about 50- to 40-fold lower than the other three preparations (Table 3). The binding constants of the highest avidity antibodies were of a similar magnitude to that determined previously for hyperimmune heterogeneous animal antiserum (Fazekas de St. Groth & Webster, 1966) or high avidity hybridoma antibody to influenza virus (Frankel & Gerhard, 1979). The ratio of IIFA to HI titres with the homologous A/USSR/90/77 antigen was in the same range for high and low avidity hybridoma antibodies, and there was no consistent relationship between avidity of IgG class antibody for homologous antigen and the relative results of HI and IIFA in tests with antigenic variants.

**DISCUSSION**

Comparison of variation in influenza virus HA detected by measuring the ability of monoclonal antibodies to bind, as well as to inhibit, the HA of naturally occurring variants has not previously been reported. Our results, obtained using a solid-phase IIFA and HI testing, show that in many cases both tests detected antigenic drift among variants of H1N1 influenza virus, presumably because variation altered the epitopes in the HA recognized by monoclonal antibodies to the extent that the antibodies no longer recognized their binding sites. The detection of antigenic variation in other cases did depend, however, on the test employed. In some instances antibody binding was detected by the solid-phase IIFA in the absence of expected levels of inhibition of haemagglutinating activity, as with monoclonal antibody preparation 22/1 and virus A/England/1/51, and preparation 70/1 with virus A/Lackland/7/78. This discrepancy was most evident with monoclonal antibody preparation 385/1 where the solid-phase immunoassay showed nearly homologous binding levels of antibodies to A/England/1/51, A/California/45/78 and A/USSR/50/79 although HI titres were reduced at least 16-fold compared to the reaction with A/USSR/90/77.

At least four explanations are envisaged for this. (i) Either the antigens or monoclonal antibodies are heterogeneous; several approaches failed to provide support for this possibility. (ii) Variation in the HA alters the affinity of the antibody–antigen reaction, which has different effects on results of IIFA and HI. Analysis of IIFA:HI titre ratios for the five monoclonal antibodies whose avidity could be determined, however, suggests that the ratio is
not a function of avidity. The possibility nevertheless remains that high avidity is the reason why the IgM monoclonal 22/1 has high binding activity in an immunoassay even with virus showing antigenic variation detectable by HI, and until more is known about the kinetics of reactions of the IgG class monoclonal antibodies with influenza HA, a role of avidity in contributing to other discrepancies we observed could not be completely ruled out. (iii) As a consequence of changed amino acid sequence, the orientation of an antigenic site may be altered with respect to an accessible part of the active site of the HA so that a monoclonal antibody bound to the antigenic site has less steric hindrance with the active site. (iv) Binding of monoclonal antibody can cause a conformational change in HA that inhibits agglutination to different degrees with different mutant viruses having altered amino acid sequence. Either of the last two hypotheses implies that the monoclonal antibodies involved (22/1, 70/1 and 385/1) are directed at epitopes which are not part of the active site region of the HA.

Comparison of immunoassay and HI results revealed an instance of variation that resulted in the biological activity of A/England/1/51 HA being inhibited to high titre by the low avidity monoclonal antibody 264/7 which bound relatively poorly to this HA as judged by the immunoassay, and a similar, but less pronounced, observation was made with antibody W18/1 and A/USSR/50/79. In this case, the antigenic site being recognized may be located at, or very close to, the active site near the tip of the HA spike, so that even when mutation in the HA reduces the affinity of the monoclonal antibody, biological activity is effectively blocked.

Previous studies have shown that some monoclonal antibodies to influenza virus HA bind to the molecule without inhibiting haemagglutination (Gerhard et al., 1980). These antibodies may bind to antigenic areas on the HA molecules not involved in haemagglutinating activity, but perhaps are analogous to those detected by immunoprecipitation reactions with antibodies in heterogeneous sera that do not contribute to HI (Schild, 1970; Six & Kasel, 1978). The present study provides the new information that variation can occur which affects the antigenic areas detected in HI while not causing a concomitant loss of reaction detected in an immunoassay.

At present, we do not know the amino acid substitutions, much less the conformational changes, in the HA of the H1N1 viruses studied here. Our results do show, however, that determination of antigenic drift with monoclonal antibodies to the HA molecule of influenza viruses may be misleading if a single serological assay is used to measure virus–antibody interactions. In general, HI appears to be more sensitive than immunoassay in detecting changes, and therefore may tend to emphasize changes that have less epidemiological significance than those detected by immunoassay, although HI is clearly more convenient than immunoassay for rapidly comparing large numbers of viruses. Our results also suggest that comparison of the ability of monoclonal antibodies to bind to, and to inhibit the biological activity of, an antigen might be helpful in understanding the relationship between the structure and the functions of the antigen.

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