THE ANALYSIS OF THE MONOCLONAL IMMUNE RESPONSE TO INFLUENZA VIRUS II. The Antigenicity of the Viral Hemagglutinin

By WALTER GERHARD*

(From The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104)

It is well established that the propensity of the influenza virus to cause recurring epidemics and pandemics is due to its extraordinary antigenic variability. Current concepts of the mechanisms underlying these antigenic variations were recently reviewed (1, 2). It is thought that major antigenic changes (antigenic shift) may result from recombinations between human and/or animal influenza strains, whereas minor antigenic changes (antigenic drift) may be due to mutations of the viral genome which affect the antigenicity of the viral surface subunits. In both instances, the antigenic changes are such that the newly arising virus strain can bypass, at least partially, the pre-existing antiviral immunity of the host population. Since only antibodies directed against the viral hemagglutinin (HA) are able to neutralize the virus and, consequently, to prevent an infection, the antigenically modified HA is a prerequisite of each new epidemic virus strain. Antigenic changes also occur in the second viral surface component, the neuraminidase (NA), but these changes seem to be of lesser importance for the survival of the virus as a human pathogen.

The structural correlate of the antigenic variability of the HA is still poorly understood. Both approaches that have previously been applied to its analysis have some obvious shortcomings. On the one hand, analysis and comparison of peptide maps of HA subunits requires relating differences to antigenicity (3-5) since antigenic variability refers only to those structures of the HA that interact with the immune system. On the other hand, the immunological approach has been hampered by the fact that sera raised in vivo against the purified HA or an appropriate hybrid virus are not necessarily monospecific with regard to the individual antigenic determinants of the HA (4, 6-9). Yet, the accurate characterization of the antigenicity of the HA, essential to an understanding of antigenic variation, will depend largely on the availability of antibodies monospecific for individual determinants.

In a previous report the production in vitro of monoclonal and consequently monospecific antiviral antibodies was described (10). This study presents the application of these antibodies to an analysis of the antigenic structure of the HA of the influenza virus PR8 [A/PR/8/34 (HON1)]. This analysis demonstrates

* Initially supported by research grants A1-08778 and CA-15822 from the U. S. Public Health Service to Dr. Norman Klinman while the author was a recipient of a Nachwuchsstipendium des Schweizerischen Nationalfonds. Supported by the National Multiple Sclerosis Society and by U. S. Public Health Service grants NS-11036 from the National Institute of Neurological Diseases and Stroke and RR-05540 from the Division of Research Resources.

1 Abbreviations used in this paper: ChHC, chicken host component; CRP, cross-reactivity patterns; HA, hemagglutinin; IAds, immunoadsorbents; NA, neuraminidase; NI, neuraminidase inhibition; RIA, radioimmunoassay; RP, reactivity patterns.
that the antigenic area of the HA of PR8 contains at least eight (groups of) antigenic determinants. One of these determinants is strain specific, whereas the others occur in a cross-reactive form on the HA of one or several A0 and A1 virus strains.

Materials and Methods

**Virus.** The influenza virus strains PR8 [A/PR/8/34(H1N1)], WSE [A/WSE/33(H1N1)], MEL [A/Mel./35(H1N1)], BEL [A/Bel./42(H1N1)], and CAM [A/Cam./46(H1N1)] were originally obtained in the form of high infectivity stocks from Dr. S. Fazekas de St. Groth, CSIRO, Molecular Genetics, Sydney, Australia. The hybrid virus Eq-PR8 [A/equine/Miami/1/63(Heq2)-A/PR/8/34(N1)] was kindly provided by Dr. R. Webster, St. Jude Children's Research Hospital, Memphis, Tenn. All viruses were grown in the allantoic cavity of 11-day-old chicken eggs and purified by adsorption to and elution from human erythrocytes followed by velocity sedimentation in a linear sucrose gradient (11). The HA activity of the virus preparations was determined as described by Fazekas de St. Groth and Webster (12).

**Immunization of Mice.** Young adult male BALB/c mice (Carworth Farms, Div. of Beckton, Dickinson & Co., N. Y., and Flow Laboratories, Inc., Rockville, Md.) were injected intraperitoneally with approximately 1,250 HA units of purified PR8 virus in phosphate-buffered saline.

**Splenocyte Cultures.** Procedures for preparing spleen cells from PR8-primed mice for the adoptive cell transfer into lethally irradiated syngeneic recipient mice and for preparing the splenic fragment cultures were as previously described (10). The culture fluid from individual splenic fragments producing antiviral antibody was harvested at 3- to 4-day intervals between the 6th and the 30th day after secondary antigenic stimulation in vitro and was pooled before being analyzed in the radioimmunassay (RIA) for the reactivity of the secreted antibodies.

**RIA.** The preparation of the viral immunoadsorbents (IAbs) (purified influenza virus covalently coupled to bromoacetyl-cellulose) and the methodology involved in the quantitation and analysis of small amounts of antiviral antibodies was as described previously (10, 13). 125I-labeled rabbit anti-mouse F(ab')2 antibody which detects all mouse immunoglobulin classes was used throughout this analysis.

**Measurement of Neutralizing Activity of Antibodies.** Virus neutralization was performed using the allantois on shell system as described by Fazekas de St. Groth et al. (14) with some modifications. Briefly, medium 199 with Hanks' salts (Flow Laboratories, Inc.) was used, supplemented with 50 μg/ml of gentamicin (Schering Diagnostics Div., Schering Corp., Port Reading, N. J.), 2 μg/ml of fungizone (E. R. Squibb & Sons, New York), 0.5% (wt/vol) of bovine serum albumin, and buffered with either 2.6 ml of 0.336 M HEPES buffer, pH 7.2 (Sigma Chemical Co., St. Louis, Mo.) or 1.5 ml 5.6% (wt/vol) of sodium bicarbonate/100 ml medium. The allantois on shell pieces were distributed into the wells of multidish disposo trays (CV-96-TC; Linbro Chemical Co., New Haven, Conn.) containing 0.2 ml HEPES-buffered medium per well. One drop (30–40 μl) of culture fluid from individual antibody-producing splenic fragments, followed by 30–40 μl of various concentrations of virus and 0.2 ml of bicarbonate-buffered medium were added to each well. The culture plates were incubated stationary at 35°C in a humidified atmosphere of O2/CO2 (93%/7%). After 3 days of incubation the culture fluid from each individual drop of shell was analyzed for the presence of HA activity as a measure of virus growth. Each titration was performed in duplicate or quadruplicate. The neutralizing activity of antibody-containing culture fluids was compared to the virus infectivity titer observed in the presence of an identical concentration of normal splenic fragment culture medium.

**Measurement of the Neuraminidase-Inhibiting (NI) Activity.** The NI test followed essentially the procedure recommended by the W. H. O. International Reference Centers for Influenza (15). Briefly, duplicate samples (75 μl) of undiluted culture fluids from individual splenic fragments that secreted antiviral antibodies were incubated for 2 h with the appropriate dilution of purified PR8 virus. Several culture fluids from splenic fragments that did not contain detectable amounts of antiviral antibody were included in each assay as controls. The rest of the procedure followed the W. H. O. recommendations exactly. A reduction of the absorbancy at 549 nm by more than 50% (compared to the mean of the control samples (0.7–1.0)) was regarded as indicating the presence of antibodies with NI activity.
Table I
Partial Degree of Interaction of Monofocal Anti-HA (PR8) Antibodies as Detected in RIA and Assignment of Reactivity Pattern

| Culture fluid no. | PR8 % | Log₂ | WSE % | Log₂ | MEL % | Log₂ | BEL % | Log₂ |
|------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| 7/251            | 100   | 0     | 111   | +0.15 | <5    | ≤4.32 | 100   | 0     |
| 8/55             | 100   | 0     | 15    | −2.74 | <2    | ≤5.54 | 28    | −1.84 |
| 7/161            | 100   | 0     | 73    | −0.45 | <3    | ≤5.06 | 21    | −2.25 |

Assigned reactivity pattern

+ + − −

* The binding of the indicated monofocal antibodies to the various IAds is expressed both as percentage (%) and a fraction on a log₂ basis (Log₂) of the homologous interaction. Entries represent averages of four samples in the case of the homologous interaction (PR8) and of three samples in the case of the heterologous interactions. A difference of 1.36 in the extent of binding of a monofocal antibody (on log₂ basis) is significant at 95% confidence limit (Student’s t test).

Results

Determination of the Reactivity Pattern (RP) of Monofocal Antibodies. Replicate samples (usually 25 μl) of the pooled culture fluid of individual antibody-producing splenic fragments were assayed in the RIA for the extent of antibody interaction with homologous and heterologous IAds. The amount of antibody in 25 μl of culture fluid (average of four determinations) which bound to the homologous IAds (PR8) was taken as the reference value (100%) of the antibody content of the given culture fluid and all heterologous antibody-IAds interactions (average of RIA performed in duplicate or triplicate) were expressed as a percentage thereof. As exemplified in Table I, heterologous interactions ranged usually from nondetectable to 100% and in some instances more than 100% of the homologous binding. The reproducibility of these partial binding values was assessed by repeated testing of various monofocal antibodies. The average standard deviation of a binding value thus obtained was, on a log₂ basis, ±0.48. These partial binding patterns were disregarded, however, for the most part of this study. Instead, simplified types of RP, all (+) or none (−), were assigned to each antibody (Table I) on the basis of its reactivity in the RIA as follows: (a) Given the minimal amount of antibody used per assay (3 ng) and given the lower limit of antibody detection in the RIA (0.2–0.3 ng), less than 10% (in general nondetectable) of the homologous interaction was scored as negative reactivity. (b) Any degree of binding that comprised more than 10% of the homologous interaction was scored as positive reactivity even though selected monofocal antibodies within each RP could be shown to differ significantly (95% confidence limit in Student’s t test) from each other with regard to their cross-reactivity (Table I). These RP were highly reproducible; only in 2 out of 72 repeat assays did the results indicate a difference of the RP of two monofocal antibodies.
**Determination of the Antibody Specificity.** Monofocal anti-PR8 antibodies were assayed in the RIA for their reactivity to the homologous virus, PR8; the hybrid virus, Eq-PR8 (both egg-grown); and the partially purified chicken host component (ChHC) as previously described (10). Since the standard PR8 virus and the parent virus used for the production of the Eq-PR8 hybrid originated from the same laboratory it was assumed that these two viruses differ antigenically only with respect to their HA, and that antibodies binding to PR8 but not to Eq-PR8 and ChHC were therefore directed against a determinant of the HA of PR8. To verify the validity of this method, various monofocal antibodies were tested in functional assays for their neutralizing or NI activity.

The splenic fragment culture fluids used in neutralization and NI tests contained at least 5 ng and 3.5 ng of antibody/25 μl, respectively (Table II). Eight antibodies with anti-NA specificity (determined on the basis of the RIA) behaved in the functional assays essentially as expected (16-18), i.e., they inhibited the enzyme activity of PR8 and exhibited only a borderline neutralizing activity. All 10 monofocal antibodies with anti-HA specificity, on the other hand, neutralized efficiently the homologous virus. However, 4 out of 22 anti-HA antibodies also inhibited the NA activity of PR8. There are several possible explanations for this unexpected NI activity of the four monofocal antibodies which scored as anti-HA antibodies in the RIA: (a) the NA of PR8 may contain a determinant not shared by the NA of the hybrid virus Eq-PR8; (b) the culture fluids may be biclonal in origin and contain, besides anti-HA antibodies, either a small quantity (<0.25 ng/25 μl) of anti-NA antibodies of high avidity (and thus of a high NI potency) or anti-NA antibodies directed against an NA determinant that was altered during preparation of the viral IADs (13); (c) certain anti-HA antibodies may be more efficient in sterically inhibiting the viral NA or may express a minor cross-reactivity with NA when a polyvalent antibody-virus interaction is possible (7, 19). Since these antibodies of questionable specificity would represent only a small fraction (roughly 12%) of the antibodies included in this analysis, they should not interfere with the overall relevance of the analysis for determinants of the HA. In the following it is assumed that all "anti-HA" antibodies (specificity determined by means of the RIA) are directed against the HA.

Table III shows the various patterns of interactions of monofocal anti-HA(PR8) antibodies with the homologous and four heterologous viruses. Several points are evident. First, 14 of the 16 theoretically possible cross-reactivity patterns [cross-reactivity patterns (CRP) are defined as RP exclusive of the homologous interaction with PR8] were realized, though at greatly differing frequency, by monofocal antibodies (Table III, first column). The analysis of an additional 131 monofocal antibodies obtained by the stimulation in vitro of PR8-primed spleen cells by heterologous viruses essentially confirmed the various RP as indicated in Table III. Thus, RP no. 15 apparently cannot be realized by BALB/c antibodies or the corresponding precursor B cells occur at very low frequency and RP no. 2 was exhibited only by 1 (antibody biclonal in origin?) out of 225 monofocal anti-PR8 antibodies tested so far. On the other hand, antibodies of RP nos. 1, 3, 4, 13, 14, and especially 16 were frequently observed and constituted roughly 80% of the anti-HA(PR8) antibodies. The data thus suggest that antibodies exhibiting either one of the pairs of reciprocal CRP tend to occur.


### Table II

**Comparison of Monofocal Antiviral Antibodies in RIA and Functional Assays**

| Immunoadsorbent | Assigned antibody specificity | Neutralization of PR8§ | Inhibition of NA activity of PR8§ |
|-----------------|------------------------------|------------------------|----------------------------------|
| PR8 | Eq-PR8 | ChHC | Anti-HA | 10 | 0 | 4 | 18 |
| + | − | − | Anti-NA | 0 | 3 | 5 | 0 |

Randomly selected monofocal antibodies were tested for their specificity in the RIA and in either one of the functional assays.

* The criteria for positive and negative reactivity in the RIA as well as the composition of the viral LAds is explained in the text. ChHC, partially purified chicken host component. Neutralization and NI were performed as described in the Materials and Methods.

§ +, more than 50% inhibition of NA activity of PR8.

### Table III

**Reactivity of Monofocal Anti-HA (PR8) Antibodies**

| RP no. | Reactivity pattern* | Number of clones† | % Antibody binding in RIA (average)§ |
|--------|---------------------|-------------------|-------------------------------------|
|        | P | W | M | B | C | WSE | MEL | BEL | CAM |
| 1      | + | + | + | + | + | 5   | 44  | 22  | 78  | 69  |
| 2      | + | − | + | + | + | 0   | 0   | 0   | 0   | 0   |
| 3      | + | + | − | + | + | 7   | 55  | <5  | 60  | 85  |
| 4      | + | + | + | + | + | 8   | 86  | 55  | <4  | 59  |
| 5      | + | + | + | + | + | 4   | 51  | 79  | 71  | <6  |
| 6      | + | + | + | + | + | 2   | <6  | <6  | 59  | 33  |
| 7      | + | + | + | + | + | 3   | <5  | 29  | <5  | 30  |
| 8      | + | + | + | + | + | 3   | <7  | 71  | 40  | <7  |
| 9      | + | + | + | + | + | 2   | 21  | <5  | <5  | 84  |
| 10     | + | + | + | + | + | 1   | 108 | <4  | 59  | <4  |
| 11     | + | + | + | + | + | 1   | 51  | 11  | <3  | <3  |
| 12     | + | + | + | + | + | 3   | <5  | <5  | <5  | 27  |
| 13     | + | + | + | + | + | 6   | <4  | <4  | 40  | <4  |
| 14     | + | + | + | + | + | 11  | <4  | 68  | <4  | <4  |
| 15     | + | + | + | + | + | 0   | 0   | 0   | 0   | 0   |
| 16     | + | + | + | + | + | 38  | <3  | <3  | <3  | <3  |

* Definition of an RP as explained in the Materials and Methods. Viral LAds: P, PR8; W, WSE; M, MEL; B, BEL; and C, CAM.
† Number of monofocal antibodies exhibiting the indicated RP.
§ Geometric mean of the fraction of the various antibodies binding to the indicated viral LAds. Expressed as percentage of the homologous interaction: PR8, 100%.

at similar frequency (such as RP nos. 3 and 14, or 4, and 13) or cannot be realized at all (such as RP nos. 2 and 15).

Second, Table III shows that, since neither antibodies reactive to a heterologous virus but nonreactive to the homologous virus nor antibodies of RP no. 15 were observed, RP nos. 9–16 must reflect the reactivity of monoclonal antibody
populations. On the other hand, RP nos. 1-8 could theoretically be due to various combinations of the aforementioned antibodies (e.g., RP no. 4 could be due to a mixture of antibodies of RP nos. 11 and 12). This is unlikely, however, for two reasons: (a) the fraction of polyclonal antibodies is estimated to be less than 10% of the monofocal antibodies included in Table III; (b) given the observed frequencies of antibodies of RP nos. 9-16 and assuming that combinations would occur randomly, roughly only 5% of the combinations of two such antibodies would actually lead to the generation of an RP of nos. 2-8. Thus, if 10% of the monofocal antibodies would be polyclonal in origin, the 62 antibodies of RP nos. 9-16 would produce less than one biclonal antibody of RP nos. 2-8.

Third, it is evident from the average amounts of the monofocal antibodies binding to the various viruses that a clear-cut difference exists between detectable and nondetectable binding of these antibodies in the RIA. (The only exception, the antibody of RP no. 11, has also been observed in other experiments not included in Table III.) The antibodies of each RP generally interacted less with the heterologous viruses than with the homologous virus. Heterologous interactions that exceeded the homologous interaction were observed, however, especially with antibodies of RP nos. 1 and 5. This is demonstrated in Table IV where 11 monofocal antibodies of RP no. 5 are grouped according to the fractional extent of cross-reaction, in such a way as to produce subgroups with standard deviations (SD) that did not significantly exceed the SD of binding values observed when monofocal antibodies were assayed repeatedly (0.48). The data of Table IV further shows that the monofocal antibodies of RP no. 5 (and probably of most other RPs defined in this study) are not a homogenous group of antibodies. Yet, the number of antibodies with slightly different reactivities to the viral HA is probably considerably smaller than the number of antibodies included in a given RP.

Discussion

The antibodies used in this analysis were produced in vitro in an adoptive transfer system. Such antibodies have previously been shown to fulfill various criteria of monoclonality (10, 20) and roughly 90% of the antibodies produced under the present experimental conditions are expected, on the basis of these arguments, to be monoclonal in origin. They thus represent individual homogeneous populations of antibody-combining sites, and hence fulfill the ultimate criterium of monospecificity. In contrast, the monospecific anti-HA antisera applied in previous studies, though specific for the given viral protein, are composed of a vast array of antibodies with specificities for an unknown number of determinants of the viral HA. They delineate, therefore, the overall antigenicity of the HA or an undefined portion thereof. Furthermore, the present finding of many individual anti-HA antibody clones implies that antisera produced in vivo, even those induced by cross-stimulation or those cross-absorbed, may still contain heterogeneous antibody populations. Thus, any variation in the relative concentration of individual antibody populations composing an antiserum may result in a different apparent specificity (9) and may yield incorrect assessments of the determinants being analyzed.

The present study demonstrates that the broad antigenic relationship of viral HA of the A0 and A1 subtype can be resolved by means of monoclonal antibodies
into many of its integral antigenic relations. If the various antibody-virus interactions observed in the RIA are defined as all (+) or none (−) types of reactions (RP) 14 antigenically different structures of the five viral HA included in this analysis can be distinguished. It seems likely, however, that the number of antigenic sites discriminated in the form of the various RP underestimates the actual number of HA determinants that can be identified by monoclonal anti-PR8 antibodies. First, the monoclonal antibodies of a given RP rarely reacted to the same extent with homologous and heterologous HA as would be expected if they shared an identical determinant. Second, the various antibodies within a given RP were heterogeneous with regard to their extent of cross-reaction, suggesting that individual antigenic sites may not represent a single, but rather a group of antigenic determinants. Henceforth, the term determinant will be used to designate the antigen structure of the heterologous HA which cross-react with the various monoclonal anti-PR8 antibodies of a given RP.

In addition to delineating a total of 13 antigenic determinants on the four heterologous viruses, all anti-PR8 antibodies included in this analysis also reacted with the homologous HA(PR8). Two alternatives must be considered in interpreting this finding with regard to the antigenic composition of the HA(PR8) itself. (a) The antibodies of each RP could be specific for an individual determinant of the HA(PR8) that occurs in an identical or cross-reactive form on one or several of the heterologous HA; the HA(PR8) would thus contain a minimum of 14 distinct antigenic determinants and each of the heterologous HA would contain, probably in addition to determinants not present on the HA of PR8, a different combination of seven HA(PR8) determinants (Table III). (b) Alternatively, various RP might merely reflect the heterogeneity of the humoral immune response to only few determinants of the homologous HA. Thus, it would be conceivable that two monoclonal antibodies contain combining sites which are identical in the structures that interact with a given determinant of the HA(PR8) but differ in those structures that are involved in the discrimination of heterologous determinants (21, 22). This type of heterospecificity at the level of individual combining sites has been observed in the interaction of homogeneous populations of antibodies with structurally dissimilar haptens (23, 24). However, since the anti-PR8 antibodies demonstrated the presence of seven cross-reactive antigenic determinants on each heterologous HA, it seems reasonable to assume that the HA(PR8) itself contains at least eight determinants,
one strain-specific and, analogous to the heterologous HA, seven cross-reactive determinants. Presently, it seems difficult either to prove or to exclude whether this latter mechanism is responsible for the generation of part of the RP demonstrated in this analysis. The observation, that various pairs of reciprocal CRP tended to occur at similar frequency such as RP nos. 4 (PWMBC: + + + - +) and 13 (+ - - + -) or not at all such as RP nos. 2 (+ - + + +) and 15 (+ + - - -) seems, however, to favor the idea that reciprocal CRP may be due to the heterogeneity of the antibody response to a single HA(PR8) determinant.

The recent studies from the laboratories of Schild, Laver, and Webster (4, 6-8) have clearly established the fact that the viral HA contains at least two (groups of antigenic determinants, one strain specific and the other common to several virus strains. Their evidence was based on the demonstration of partial identity in immunoprecipitation tests between different virus strains and monospecific anti-HA antisera and on the ability to remove antibodies reactive to either determinant(s) completely and selectively by adsorption of the serum with the appropriate virus strain. Furthermore, those studies demonstrated that in the course of the antigenic drift, i.e. the gradual change of the antigenicity of the HA of successively arising interpandemic virus strains, the strain-specific HA determinant(s) underwent major antigenic changes, whereas other (common) determinant(s) remained unchanged.

The present analysis further extends the above mentioned observations with regard to the so-called common HA determinant(s). This latter determinant could be dissected by means of the monoclonal anti-PR8 antibodies into several (7-13) distinguishable determinants. Each of these determinants (with the possible exception of one) occurred in an antigenically changed form on two of several of the virus strains included in the analysis. Thus, the partially shared determinants undergo antigenic changes similarly to the strain-specific determinant(s). It is interesting, however, that the antigenic variability in the former determinants seems to be rather unordered and does not reveal any evidence of a relationship between the observed antigenic changes and the year when the given virus strain was originally isolated. Thus, the virus CAM (year of original isolation, 1945) shared with PR8 (1934) an identical number of cross-reactive determinants and interacted in the RIA with roughly the same number of anti-PR8 antibodies as did BEL (1942) or MEL (1935). Experiments are currently underway to further delineate this set of partially shared determinants by means of monoclonal antibodies raised against other A0 virus strains. This set of partially shared determinants may represent a highly variable protein structure from which originate the major changes that lead to the formation of the various strain-specific determinants.

The well established broad cross-reactivity of A0 and A1 HA (2, 7, 8, 21, 25, 26) is due to this set of partially shared determinants. Of particular interest is the determinant delineated by the antibodies of RP no. 1. This determinant occurs in a cross-reactive form on all five virus strains used in this analysis. Since A0 and A1 virus strains contain the same type of NA two antibodies of RP no. 1 were also tested in the NI assay; neither of them inhibited the enzyme activity of PR8. It remains, however, to be shown whether this common determinant is also present on other A0 and A1 virus strains and especially in view of its potential significance in the selection of a vaccine strain or vaccination schedule, whether
antibodies of RP no. 1 exhibit also a corresponding cross-reaction in functional assays such as virus neutralization (7).

In conclusion, the present study demonstrates, firstly, that the application of monoclonal antibodies to the antigenic analysis of the HA (and probably of any complex antigen) is feasible and, secondly, that at the level of monoclonal antibodies many individual HA determinants can be discriminated. On the other hand, this study cannot specify whether these determinants are spatially separated or overlapping structures of a single or several antigenic sites nor where on the HA spike the various determinants are located. However, with regard to the solution of these latter questions which certainly cannot be achieved by immunological methods alone, the availability of monoclonal antibodies is likely to play a crucial role.

Summary

The antigenicity of the hemagglutinins (HA) of five influenza viruses of the A0 and A1 subtypes has been analyzed by means of monoclonal antibodies of murine origin produced in vitro. Secondary monoclonal anti-HA(PR8) antibodies were able to differentiate 14 antigenic determinants (or groups of determinants) on the HA of five influenza virus strains of the A0 and A1 subtypes. Taking into account that certain pairs of determinants delineated on heterologous HA may reflect the heterogeneity of the humoral immune response to a single homologous determinant, the presence of at least eight determinants (host cell-derived determinants not included) on the homologous HA of PR8 and probably on the HA of influenza viruses in general is postulated.

Three types of HA-determinants of A0 and A1 influenza virus strains could be distinguished: strain-specific, partially shared, and determinant(s) common to all five virus strains tested. Roughly 40, 55, and 5%, respectively, of the secondary anti-PR8 antibodies of BALB/c mice were directed against determinants belonging to either of the three types.

I am grateful to Dr. Norman Klinman, in whose laboratory this work was initiated, for his support and advice; and to Doctors Vivian Braciale, Thomas Braciale, and Susan Pierce for initial help and discussions. I thank Dr. H. Koprowski for his support and encouragement during the course of this study. The excellent technical assistance of Mrs. Maureen Carey is gratefully acknowledged.

Received for publication 6 May 1976.

References

1. Webster, R. G., and W. G. Laver. 1971. Antigenic variation in influenza virus biology and chemistry. Prog. Med. Virol. 13:271.
2. Dowdle, W. R., M. T. Colman, and M. B. Gregg. 1974. Natural history of influenza type A in the United States, 1957-1972. Prog. Med. Virol. 17:91.
3. Laver, W. G., and R. G. Webster. 1968. Selection of antigenic mutants of influenza viruses. Isolation and peptide mapping of their hemagglutinating proteins. Virology. 34:193.
4. Laver, W. G., J. C. Downie, and R. G. Webster. 1974. Studies on antigenic variation in influenza virus. Evidence for multiple antigenic determinants of the hemagglutinin subunits of A/Hong Kong/68 (H3N2) virus and the A/England/72 strains. Virology. 59:230.
5. Webster, R. G., W. G. Laver, and B. Tumova. 1975. Studies on the origin of pandemic influenza viruses. V. Persistence of Asian influenza virus hemagglutinin (H2) antigen in nature? Virology. 67:534.

6. Schild, G. C., J. S. Oxford, W. R. Dowdle, M. Coleman, M. S. Pereira, and P. Chakraverty. 1974. Antigenic variation in current influenza A viruses: evidence for a high frequency of antigenic "drift" for the Hong Kong virus. Bull. W. H. O. 51:1.

7. Schild, G. C. 1970. Studies with antibody to the purified hemagglutinin of an influenza A0 virus. J. Gen. Virol. 9:191.

8. Virelizier, J-L., R. Postlethwaite, G. C. Schild, and A. C. Allison. 1974. Antibody responses to antigenic determinants of influenza virus hemagglutinin. I. Thymus dependence of antibody formation and thymus independence of immunological memory. J. Exp. Med. 140:1559.

9. Laver, W. G., J. C. Downie, and R. G. Webster. 1976. Diversity of the antibody response to the different antigenic determinants on the hemagglutinin subunits of influenza viruses. J. Immunol. 116:336.

10. Gerhard, W., T. J. Braciale, and N. R. Klinman. 1975. The analysis of the monoclonal immune response to influenza virus. I. Production of monoclonal anti-viral antibodies in vitro. Eur. J. Immunol. 5:720.

11. Laver, W. G. 1969. In Fundamental Techniques in Virology. K. Habel and N. P. Salzman, editors. Academic Press, Inc., New York. 82.

12. Fazekas de St. Groth, S., and R. G. Webster. 1966. Disquisitions on original antigenic sin. I. Evidence in man. J. Exp. Med. 124:331.

13. Braciale, T. J., W. Gerhard, and N. R. Klinman. 1976. Analysis of the humoral immune response to influenza virus in vitro. J. Immunol. 116:827.

14. Fazekas de St. Groth, S., J. Withell, and K. J. Lafferty. 1958. An improved assay method for neutralizing antibodies against influenza viruses. J. Hyg. 56:415.

15. Aymard-Henry, M., M. T. Coleman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster. 1973. Influenzavirus neuraminidase and neuraminidase-inhibition test procedures. Bull. W. H. O. 48:199.

16. Webster, R. G., and W. G. Laver. 1967. Preparation and properties of antibody directed specifically against the neuraminidase of influenza virus. J. Immunol. 99:49.

17. Kilbourne, E. D., W. G. Laver, J. L. Schulman, and R. G. Webster. 1968. Antiviral activity of antiserum specific for an influenza virus neuraminidase. J. Virol. 2:281.

18. Compans, R. W., N. J. Dimmock, and H. Meier-Ewert. 1969. Effect of antibody to neuraminidase on the maturation and hemagglutinating activity of an influenza A2 virus. J. Virol. 4:528.

19. Easterday, B., W. G. Laver, H. G. Pereira, and G. C. Schild. 1969. Antigenic composition of recombinant virus strains produced from human and avian influenza A viruses. J. Gen. Virol. 5:233.

20. Klinman, N. R. 1971. Regain of homogeneous binding activity after recombination of chains of "monofocal" antibody. J. Immunol. 106:1330.

21. Fazekas de St. Groth, S. 1967. Cross recognition and cross reactivity. Cold Spring Harbor Symp. Quant. Biol. 32:525.

22. Fazekas de St. Groth, S. 1969. New criteria for the selection of influenza vaccine strains. Bull. W. H. O. 41:651.

23. Varga, J. M., W. H. Konigsberg, and F. F. Richards. 1973. Antibodies with multiple binding functions. Induction of single immunoglobulin species by structurally dissimilar haptenns. Proc. Natl. Acad. Sci. U. S. A. 70:3289.

24. Michaelides, M. C., and H. N. Eisen. 1974. The strange cross-reaction of menadione (vitamin K3) and 2,4-dinitrophenyl ligands with a myeloma protein and some conventional antibodies. J. Exp. Med. 140:687.
25. Fazekas de St. Groth, S. 1969. The antigenic subunits of influenza viruses. II. The spectrum of cross reactions. *J. Immunol.* 103:1107.
26. Baker, N., N. O. Stone, and R. G. Webster. 1973. Serological cross-reactions between the hemagglutinin subunits of HON1 and HIN1 influenza viruses detected with "monospecific" antisera. *J. Virol.* 11:137.