JOURNAL OF VIROLOGY, Jan. 1986, p. 124–128
0022-538X/86/010124-05$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 57, No. 1

Comparative Analyses of the Specificities of Anti-Influenza Hemagglutinin Antibodies in Human Sera

M.-L. WANG,[1]† J. J. SKEHEL,[1]* AND D. C. WILEY[2]

Division of Virology, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom,[1] and Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138[2]

Received 23 August 1985/Accepted 1 October 1985

Estimates of the variety of specificities of anti-influenza hemagglutinin antibodies in postinfection human sera taken between 1969 and 1971 and in 1978 were made by using Fab fragments of defined monoclonal antibodies in competitive virus-binding assays. The results obtained with the sera taken between 1969 and 1971 indicated that different sera contained antibodies with different ranges of specificities, whereas the 1978 sera mainly contained a broad range of antibodies. The results are discussed in relation to the mechanism of antigenic drift in influenza virus, the commonly observed antigenic heterogeneity of influenza virus isolates, and the efficacy of antiinfluenza vaccination.

Antigenic variations among the hemagglutinins (HAs) of Hong Kong (H3 subtype) influenza viruses result from differences in amino acid sequences at a number of positions in the peripheral globular regions of the molecules (22, 23). In natural antigenic variants isolated from infected humans between 1968 and 1983, 86 (21%) amino acid substitutions were observed in the HA_1 polypeptides, which exclusively make up these globular regions, as compared with 6 (2%) in the HA_2 components, which form the base and central stem of the molecule (1, 16, 21). This drift in amino acid sequence and antigenicity is commonly considered (for reviews, see references 17 and 22) to involve selection by immunological pressures of mutants which arise during virus replication. In attempts to understand in more detail the process of antigenic drift in the HAs of viruses isolated from humans, we estimated the contribution to the selection pressures of antibodies of different specificities by analyzing the anti-HA antibody populations of postinfection human sera. Specifically, in the experiments described in this report, we used in competitive binding assays ^{125}I-labeled Fab fragments prepared from anti-HA monoclonal antibodies of precisely defined specificities to investigate the range of specificities exhibited by the antibodies present in two collections of human sera taken between 1969 and 1971 and in 1978.

MATERIALS AND METHODS

Sera. The two collections of human sera used were taken between 1969 and 1971 and in 1978 from individuals between the ages of 2 and 32 and recovering from influenza virus infections. The sera were kindly provided by M. S. Pereira of the Virus Reference Laboratory, Colindale, London, United Kingdom.

Monoclonal antibodies. BALB/C mice were immunized by intraperitoneal injection of 8,000 HA units of purified virus. Eight weeks later they received 32,000 HA units of virus intraperitoneally and intravenously. Three days later their spleens were removed, and the cells were fused with SP2/0-Ag14 myeloma cells (15) as described by Kohler and Milstein (7, 8). Hybrid cell culture conditions were based on those described by Fazekas de St. Groth and Scheidegger (4).

Antibody purification and Fab fragment preparation. Immunoglobulins were purified from ascitic fluids by affinity chromatography on protein A-Sepharose (3). Immunoglobulin G from 5 ml of ascitic fluid was adsorbed to a 5-ml column containing phosphate-buffered saline (PBS) (pH 7.2), eluted with 0.1 M citrate (pH 4.0), dialyzed against PBS, and concentrated to 2 to 4 mg/ml. Purified immunoglobulin G was digested for 1 to 3 h with 1% (wt/wt) papain preactivated by incubation in 0.01 M cysteine. Fab fragments were purified from the digestion products by using a protein A-Sepharose column. Purified immunoglobulin G and Fab fragments were analyzed by polyacrylamide gel electrophoresis in buffers containing sodium dodecyl sulfate. Binding constants were determined as described by Frankel and Gerhard (5) by using purified virus as antigen and ^{125}I-labeled rabbit antimouse immunoglobulin G (light chain) for complex detection. Fab fragments were iodinated with IODOGEN (Pierce Chemical Co.) (10). Fab fragments (20 to 40 μg) in 50 μl of PBS (pH 7.2) were incubated with 1 mCi of Na^{125}I (Amersham Corp.) for 30 min at room temperature in a tube coated with 100 μg of IODOGEN. Fab fragments were dialyzed against PBS. The specific activities obtained were between 3.77 and 12.35 Ci/g of protein.

Viruses. X-31 (H3N2) recombinant influenza virus was used as the representative 1968 virus (6), and A/Texas/1/77 (H3N2) was used as the representative 1977 virus. Antigenic variants used to define the specificities of the monoclonal antibodies were obtained by mixing equal volumes of allantoic fluid containing virus and undiluted ascitic fluid containing monoclonal antibodies and using the mixture as the inoculum. Variants obtained were cloned by limit dilution. Viruses were purified as described previously (18).

Nucleotide sequence analyses. Sequences of the genes for the HAs of the monoclonal antibody-selected antigenic variants were determined by the dideoxynucleotide chain-terminating procedure of Sanger et al. (14) as described elsewhere (2).

* Corresponding author.
† Present address: Hubei Academy of Medical Sciences, Wuhan, Hubei, China.

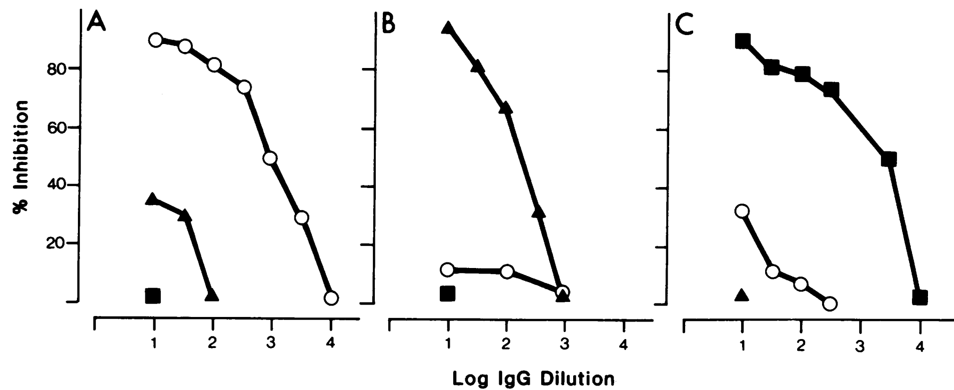

FIG. 1. Competition for binding to X-31 influenza virus between ^{125}I-labeled Fab fragments of monoclonal antibodies and monoclonal antibodies of different specificities. The properties of the monoclonal antibodies used are given in Table 1. (A) Competition with ^{125}I-Fab HC3; (B) competition with ^{125}I-Fab HC19; (C) competition with ^{125}I-Fab HC31. Symbols: ○, competition by HC3 antibody, which recognizes HA_1 residue 144; ▲, competition by HC19 antibody, which recognizes HA_1 residue 157; ■, competition by HC31 antibody, which recognizes HA_1 residue 198. IgG, Immunoglobulin G.

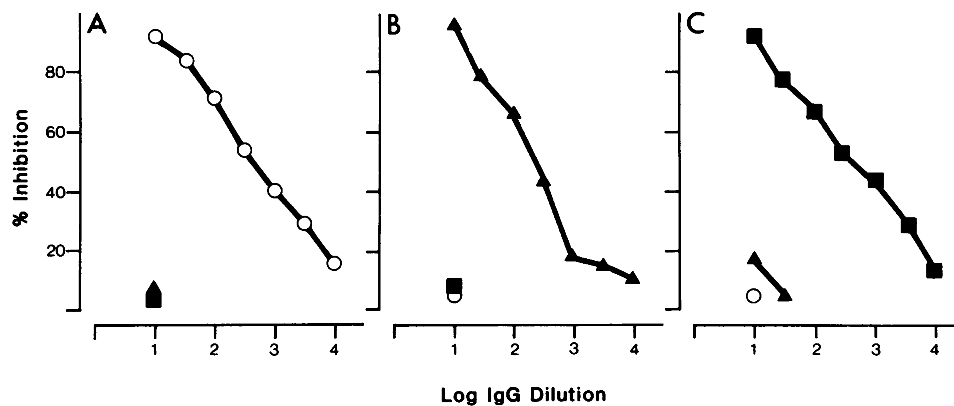

FIG. 2. Competition for binding to A/Texas/1/77 influenza virus between ^{125}I-labeled Fab fragments of monoclonal antibodies and monoclonal antibodies of different specificities. The properties of the monoclonal antibodies used are given in Table 1. (A) Competition with ^{125}I-Fab HC59; (B) competition with ^{125}I-Fab HC87; (C) competition with ^{125}I-Fab HC92. Symbols: ○, competition by HC59 antibody, which recognizes HA_1 residue 198; ▲, competition by HC87 antibody, which recognizes HA_1 residue 144; ■, competition by HC92 antibody, which recognizes HA_1 residue 78. IgG, Immunoglobulin G.

Radioimmunoassays. Microtiter plates were coated with purified virus (200 HA units per 40 μl of PBS per well) for 16 h at 4°C. They were then washed four times with PBS containing 2% (wt/vol) bovine serum albumin. Competitive binding assays involved incubating iodinated Fab fragments in 40 μl of PBS containing 2% (wt/vol) bovine serum albumin per microtiter plate well with serial dilutions of immunoglobulins or sera which had been predigested with *Vibrio cholerae* neuraminidase. After 2 h of incubation at 37°C, the plates were washed three times with PBS containing 2% (wt/vol) bovine serum albumin and 0.05% Tween 20 and then eight times with H_2O, and the remaining radioactivity was determined. The percent inhibition was calculated as $[1 - (c/t)] \times 100$, where c and t represent the radioactivity bound in the presence and absence of competing antibody, respectively.

RESULTS

Viruses. Viruses of the H3 subtype were first isolated from widespread epidemics of influenza in 1968 and later from pandemics in 1972 (prototype virus, A/England/42/72) and

TABLE 1. Monoclonal antibody specificities and affinities

HA	Fab	Amino acid sequence changes[a]	Binding constant (10^7 M^{-1})[b]
X-31 (1968)	HC3	GGT → GAT, G → D, HA_1 residue 144	3.0
	HC19	TCA → TTA, S → L, HA_1 residue 157	2.6
	HC31	GCA → GGA, A → E HA_1 residue 198	2.8
A/Texas/1/77 (1977)	HC87	GAT → GGT, D → G, HA_1 residue 144	3.4
	HC59	GCA → GAA, A → E, HA_1 residue 198	2.7
	HC92	GGC → GTT, G → V, HA_1 residue 78	2.9

[a] Amino acid sequence changes in the HAs of antigenic variants which define the specificities of monoclonal antibodies used in the radioimmunoassays.

[b] Determined as described by Frankel and Gerhard (5).

TABLE 2. Competition for binding to X-31 influenza virus between ^{125}I-labeled Fab fragments of monoclonal antibodies and immunoglobulins in human sera

Fab	Reciprocal of indicated serum dilution at which ^{125}I-Fab binding to virus was inhibited by 40%																			
	389	167	195	384	349	26	286	12	289	10	173	365	178	14	3	27	6	24	386	361
HC3(144)							640						40	40	80	80	80	80	80	160
HC31(198)			80	80	80	80		40	40	80	80	80								
HC19(157)	20	80						40	60	40	160	160	20	80	20	20	80	80	160	40

TABLE 3. Competition for binding to A/Texas/1/77 influenza virus between ^{125}I-labeled Fab fragments of monoclonal antibodies and immunoglobulins in human sera

Fab	Reciprocal of indicated serum dilution at which ^{125}I-Fab binding to virus was inhibited by 40%																				
	1680	171	1189	1201	1205	142	155	134	1541	1169	153	1185	168	152	1187	1487	1220	1338	1356	1125	143
HC87(144)					20	160	160	1,280	1,280	10	10	10	10	10	20	20	20	20	20	40	40
HC59(198)	10	10	40	40						10	10	10	10	40	10	20	20	40	40	10	10
HC92(78)	320	640	80	320	320	160	320	320	320	10	80	320	640	320	40	20	80	80	320	10	20

1975 (A/Victoria/3/75). Since then, the incidence of serious outbreaks of influenza has been low, with the only significant epidemic occurring in 1980 to 1981 (A/Bangkok/1/79) and appearing to be restricted to the United States (for a review, see references 12 and 17). We analyzed sera obtained from patients with influenza in 1969 to 1971 and in 1978, at which times viruses antigenically similar to A/Aichi/2/68 and A/Texas/1/77 were commonly isolated.

Specificities of the standard Fab fragments. To obtain information on the specificities of anti-HA antibodies in sera, we used a competitive binding assay which involves estimating the binding to purified influenza virus of ^{125}I-labeled Fab fragments, prepared from mouse anti-HA monoclonal antibodies, in the presence of the sera under analysis. Fab fragments from three monoclonal antibodies against X-31 HA and three monoclonal antibodies against A/Texas/1/77 HA were used. Their specificities were determined (i) by a radioimmunoassay (Fig. 1 and 2), which indicated that the antibodies recognized nonoverlapping determinants on the respective HAs, and (ii) by an analysis of the complete nucleotide sequences of the genes for the HAs of the antigenic variants selected for growth in the presence of the different monoclonal antibodies. The three anti-X-31 antibodies recognized residues 144, 157, and 198 of HA_1 in antigenic region A and two distinct areas of region B, respectively (2, 23), and the three anti-A/Texas/1/77 antibodies recognized residues 78, 144, and 198 of HA_1 in regions E, A, and B, respectively (Table 1). It should be noted that although antibodies HC31(198) and HC59(198) recognized the same residues in X-31 and A/Texas/1/77 HAs, respectively, these residues were not antigenically equivalent in the different HAs, and the antibodies only reacted with the homologous antigens.

Radioimmunoassays. The results of the radioimmunoassays of the sera taken between 1969 and 1971 are shown in Table 2. They indicated that when a 40% inhibition of ^{125}I-Fab binding was used as an arbitrary minimum inhibition at any serum dilution, 33% of the sera contained antibodies of all three specificities tested, 50% lacked antibodies of only one specificity tested, and 17% appeared to lack antibodies of two of three specificities tested. For the 1977 virus and the 1978 sera (Table 3), 78% of the sera contained antibodies of all three specificities tested, and the remaining 22% lacked antibodies of only one specificity tested.

DISCUSSION

We attempted in these experiments to compare the antibody responses of different individuals to influenza virus by determining whether sera taken from them after infection contained antibodies of certain specificities. The results presented do not provide information on the actual range of anti-HA antibody specificities present in the sera, nor do they represent quantitative estimates of the relative abundance in sera of antibodies with different specificities. However, since they indicate that sera differ in their abilities to inhibit the binding to virus of different Fab fragments from monoclonal antibodies, they allow the simple conclusion that individuals respond differently to infection by producing antibodies with different ranges of specificities; this was most clearly seen in the results obtained with sera taken in the early years of Hong Kong influenza (1969 to 1971). Of these sera, only 33% contained antibodies of all three specificities tested, a further 50% had antibodies of two of the specificities tested, and the remaining 17% had antibodies of only one of the specificities tested. Since the numbers of sera negative in tests with any one specific Fab fragment were about equal and since the affinities of the Fab fragments for the HAs were also similar (Table 1), it appears that no single antigenic region of the molecule was generally recognized as immunodominant. The results obtained with sera taken in 1978 suggest that the majority contained a broad range of antibody specificities and, consequently, the possibility that this may have contributed to the decrease in the number of significant outbreaks of disease since 1975. This possibility was not directly addressed in these studies, however, primarily because of the restricted range of specificities of the Fab fragments used in the tests and because direct estimates of the infectivity-neutralizing capacity of the sera were not made.

Other observations which suggest variations in the range of specificities of anti-HA antibodies in individual animals have been made before. For example, analyses of the frequency of isolation of cloned cells producing anti-HA antibodies of a particular specificity indicated variations in the antibody repertoire of individual mice (19, 20). Variations have also been frequently observed in the specificities of anti-HA antibodies in individual postinfection ferret sera, commonly used as a source of strain-specific reference

TABLE 2—*Continued*

357	1	7	30	364	202	166	2	28	388	8	4	15	9	22	355	144	163	128	No. of sera negative (%)
160	40	80	80	160	1,280	10	80	80	80	80	80	80	80	80	160	320	640	1,280	11/39 (28.2)
	40	20	80	40	40	40	20	20	20	40	40	80	80	80	80	80	20	80	12/39 (30.8)
80						40	40	80	320	40	40	10	40	160	160	20	60	60	10/39 (25.6)

TABLE 3—*Continued*

1670	167	1442	1280	151	133	136	139	141	156	157	174	1526	172	135	145	1588	1657	1640	1548	No. of sera negative (%)
40	40	40	40	80	80	160	160	160	160	640	1,280	1,280	1,280	1,280	1,280	1,280	1,280	1,280	1,280	4/41 (9.75)
10	10	10	40	10	10	10	10	20	40	10	10	10	10	10	10	10	40	80	160	5/41 (12.20)
640	640	640	320	40	160	40	160	10	10	320	80	80	320	640	640	1,280	80	1,280	640	0/41 (0)

antibodies for both haemagglutination inhibition tests and competitive binding radioimmunoassays, such as that used here (data not shown). There are also indications of restricted antibody populations in human sera deduced from results obtained in radial hemolysis assays of their reactions with monoclonal antibody-selected antigenic variants (11). In that study of sera taken in 1979, up to 41% of adults and 58% of children failed to recognize monoclonal antibody-selected variants of A/Texas/1/77, the HAs of which presumably contained only single amino acid substitutions. These results, therefore, suggest considerably more limited ranges of antibody specificities than those observed here in the sera taken in 1978.

What are the consequences of these observations for considerations of the processes involved in antigenic drift? A number of mechanisms have been proposed in attempts to correlate the observed frequency of isolation of antigenic variants in the presence of monoclonal antibodies (24) with the frequency of occurrence of epidemiologically significant antigenic variants which would have the capacity to reinfect all members of the population. The results on which they are based mainly indicate restricted antibody responses to infection and have led to two suggestions. In the first, it is considered that certain antigenic determinants are dominant and, as a consequence, the rate at which these dominant determinants change dictates the overall rate of antigenic drift. Observations of highly restricted ranges of anti-HA antibody specificities in human (11) and animal (9) sera certainly suggest that on occasion only single antigenic determinants are recognized and, therefore, support this proposition. The results reported here, however, indicate that even after primary infection a spectrum of anti-HA responses can be found in the human population. They therefore suggest that the generation of antigenic variants with the ability to reinfect all members of the population would involve changes in at least three antigenic regions, a proposition made before on the basis of amino acid sequence differences in the HAs of Hong Kong viruses isolated in the epidemic years of 1968, 1972, and 1975 (23). In this case, epidemiologically significant antigenic variants would arise by a process of sequential change during a series of reinfections of individuals with restricted ranges of antibodies. The frequency with which they occurred would depend on the proportions of the population with different ranges of antibody specificities.

Finally, two characteristics of influenza viruses warrant consideration together with the results presented here. Analyses of influenza viruses isolated from humans with anti-HA monoclonal antibodies indicate that at anytime the viruses in circulation are antigenically heterogeneous (e.g., 13). This heterogeneity may be a reflection of differences in the responses of individuals to infection and the consequent differences in the selection pressures which they generate. It has also been observed that protective immunity is developed in considerably less than 100% of influenza vaccinees (between 50 and 75%, depending on the study), although the vaccines used are immunogenically potent. If the immune response to the noninfectious vaccines used varies with the individual vaccinee in a similar fashion to the responses to infection reported here, then the inefficiency of vaccination may be a direct consequence.

ACKNOWLEDGMENTS

We thank A. Douglas, D. Stevens, R. Gonsalves, and G. White for assistance.

We acknowledge support from Public Health Service grant AI 13654 (to D.C.W.) from the National Institutes of Health and grant 771398 (to D.C.W. for computing hardware) from NSFPC. M.-L. Wang was supported by a fellowship from the Wellcome Trust.

LITERATURE CITED

1. **Both, G. W., M. J. Sleigh, N. J. Cox, and A. P. Kendal.** 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J. Virol. **48:**52–60.
2. **Daniels, R. S., A. R. Douglas, J. J. Skehel, and D. C. Wiley.** 1983. Analysis of the antigenicity of influenza haemagglutinin at the pH optimum of virus-mediated membrane fusion. J. Gen. Virol. **64:**1657–1662.
3. **Ey, P. L., S. J. Prowse, and C. R. Jenkin.** 1978. Isolation of pure IgG, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry **15:**429–436.
4. **Fazekas de St. Groth, S., and D. Scheidegger.** 1980. Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods **35:**1–21.
5. **Frankel, M. E., and W. Gerhard.** 1979. The rapid determination of binding constants for antiviral antibodies by a radioimmuno-

assay. An analysis of the interaction between hybridoma proteins and influenza virus. Mol. Immunol. **16**:101–106.
6. **Kilbourne, E. D.** 1969. Future influenza vaccines and the use of genetic recombinants. Bull. W.H.O. **41**:643–645.
7. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
8. **Kohler, G., and C. Milstein.** 1976. Derivation of specific antibody-producing tissue culture and tumour lines by cell fusion. Eur. J. Immunol. **6**:511–519.
9. **Laver, W. G., G. M. Air, and R. G. Webster.** 1981. Mechanism of antigenic drift in influenza virus. Amino acid sequence changes in an antigenically active region of Hong Kong (H3N2) influenza virus haemagglutinin. J. Mol. Biol. **145**:339–361.
10. **Markwell, M. A. K., and C. F. Fox.** 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide binding or reversible chemical cross-linking. J. Virol. **33**:152–166.
11. **Natali, A., J. S. Oxford, and G. C. Schild.** 1981. Frequency of naturally occurring antibody to influenza virus antigenic variants selected *in vitro* with monoclonal antibody. J. Hyg. **87**:185–190.
12. **Pereira, M. S.** 1982. Persistence of influenza in a population, Symp. Soc. Gen. Microbiol. **33**:15–37.
13. **Pereira, M. S., P. Chakraverty, P. Cunningham, and R. G. Webster.** 1985. The use of monoclonal antibodies for the antigenic analysis of influenza A viruses. Bull. W.H.O. **63**:265–272.
14. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
15. **Schulman, M., C. D. Wilde, and G. Kohler.** 1978. A better cell line for making hybridomas secreting specific antibodies. Nature (London) **276**:269–270.
16. **Skehel, J. J., R. S. Daniels, A. R. Douglas, and D. C. Wiley.** 1983. Antigenic and amino acid sequence variations in the haemagglutinins of type A influenza viruses recently isolated from human subjects. Bull. W.H.O. **61**:671–676.
17. **Skehel, J. J., A. R. Douglas, I. A. Wilson, and D. C. Wiley.** 1982. Antigenic variation in the influenza A (Hong Kong) viruses, Symp. Soc. Gen. Microbiol. **33**:215–226.
18. **Skehel, J. J., and G. C. Schild.** 1971. The polypeptide composition of influenza A viruses. Virology **44**:396–408.
19. **Staudt, L. M., and W. Gerhard.** 1983. Generation of antibody diversity in the immune response of Balb/c mice to influenza virus haemagglutinin. J. Exp. Med. **157**:687–704.
20. **Underwood, P. A.** 1984. An antigenic map of the haemagglutinin of the influenza Hong Kong subtype (H3N2) constructed using mouse monoclonal antibodies. Mol. Immunol. **21**:663–671.
21. **Verhoeyen, M., R. Fang, W. Min Jou, R. Dvos, D. Buylebroeck, E. Saman, and W. Fiers.** 1980. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. Nature (London) **286**:771–776.
22. **Webster, R. G., W. G. Laver, G. M. Air, and G. C. Schild.** 1982. Molecular mechanisms of variation in influenza viruses. Nature (London) **296**:115–119.
23. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature (London) **289**:373–378.
24. **Yewdell, J. W., R. G. Webster, and W. U. Gerhard.** 1979. Antigenic variation in three distinct determinants of an influenza type HA molecule. Nature (London) **279**:246–248.