

Electron Microscopy of Influenza Haemagglutinin-Monoclonal Antibody Complexes

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The molecular locations of antibody binding sites on the haemagglutinin of influenza virus X-31 were investigated by electron microscopy of haemagglutinin-monoclonal antibody complexes. Evidence was obtained for different sites of binding of different antibodies and direct correspondence was observed between these sites and the locations of antigenic sites A, B, and E (D. C. Wiley, I. A. Wilson, and J. J. Skehel (1981). *Nature (London)* **289**, 373-378) defined by determining the amino acids recognized by the specific antibodies.

INTRODUCTION

Haemagglutinins are glycoprotein components of influenza virus membranes which bind virus particles to cells and in addition appear to be involved in the membrane fusion which allows transfer of the virus transcription complex into cells (for reviews see Compans and Klenk, 1979; Ward, 1982). Antibodies against haemagglutinins neutralize virus infectivity and studies on antigenicity using monoclonal antibodies indicate that haemagglutinins contain a small number of nonoverlapping antibody binding regions (Gerhard *et al.*, 1981; Webster *et al.*, 1980; Jackson *et al.*, 1982). Analyses of the amino acid sequences of haemagglutinins of antigenic variants indicate that antibodies of different specificities recognize different amino acid residues in the distal region of the haemagglutinin molecule (Laver *et al.*, 1979; 1980; Both and Sleigh, 1981; Caton *et al.*, 1982; Daniels *et al.*, 1983). The amino acids recognized have generally been assumed to contribute to the particular antibody binding site. We present here evidence obtained directly by electron microscopy of haemagglutinin-antibody complexes that this is the case for anti-

bodies recognizing amino acids in sites A, B, and E (Wiley *et al.*, 1981).

METHODS

Haemagglutinin preparation. Soluble preparations of the haemagglutinin (BHA) from X-31 (H3N2) (Kilbourne, 1969) were made by bromelain digestion of purified virus particles as described by Brand and Skehel (1972).

Monoclonal antibody preparation. Balb/c mice were immunized by intraperitoneal injection of 8000 HA units of purified virus. Eight weeks later they received 32,000 BHA units of virus intraperitoneally and intravenously. Three days later their spleens were removed and the cells fused with SP2/0-Ag14 myeloma cells (Shulman *et al.*, 1978) as described by Kohler and Milstein (1975, 1976). Hybrid cell culture conditions were based on those described by Fazekas de St. Groth and Scheidegger (1980). Antibodies were purified by affinity chromatography on protein A-sepharose CL-4B (Pharmacia).

Nucleotide sequence analyses. Sequences were determined using the dideoxynucleotide chain terminating procedure of Sanger *et al.* (1977). Each 10-μl reaction contained Tris-Cl pH 8.3, 0.05 M; MgCl₂, 0.012 M; dithiothreitol, 0.02 M; dATP, dCTP, dGTP,

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TABLE 1

AMINO ACID CHANGES IN THE HAEMAGGLUTININS OF ANTIGENIC VARIANTS SELECTED WITH MONOCLONAL ANTIBODIES

Antibody	Variant	Nucleotide change	Amino acid change
HC3	V3	508 G → A	Gly → Asp 144
HC19	V19	547 C → T	Ser → Leu 157
HC31	V31	670 C → T	Ala → Val 198
HC45	V45	264 G → A	Asp → Asn 63

Note. Antigenic variants of X-31 were obtained by mixing equal volumes of allantoic fluid-containing virus and undiluted ascitic fluid-containing monoclonal antibodies and using the mixture as inoculum. Variants obtained were cloned by limit dilution. Nucleotide sequences were determined as described under Methods. These were the only changes detected in the HA₁ regions of the HA genes.

dTTP, 0.0004 M; human placenta RNase inhibitor (Bethesda Research Labs.) 3 units; reverse transcriptase, 5 units (Life Sciences); virus RNA, 7 µg; and either ddATP, ddCTP, ddGTP, or ddTTP, 0.00025 M. After 120 min at 42° products were analyzed on polyacrylamide gels containing 8% acrylamide. Reactions were primed using ³²P-5'-labeled synthetic oligodeoxynucleotides, 5 pmol (Patel *et al.*, 1982) numbered according to the sequence of X-31 haemagglutinin cDNA (Verhoeyen *et al.*, 1980): 5-AAAGCAGGG-14; 191-TGC-TACTGAGCT-202; 345-CGCAGCAAAG-354; 498-GCAAAAGGG-502; 623-TCA-CCACCG-632; 777-TGGACAATA-786. The nucleotide sequence changes determined in the HA₁ regions of appropriate antigenic variants, which serve to define the specificity of the monoclonal antibodies used, are given in Table 1.

Electron microscopy. BHA was diluted

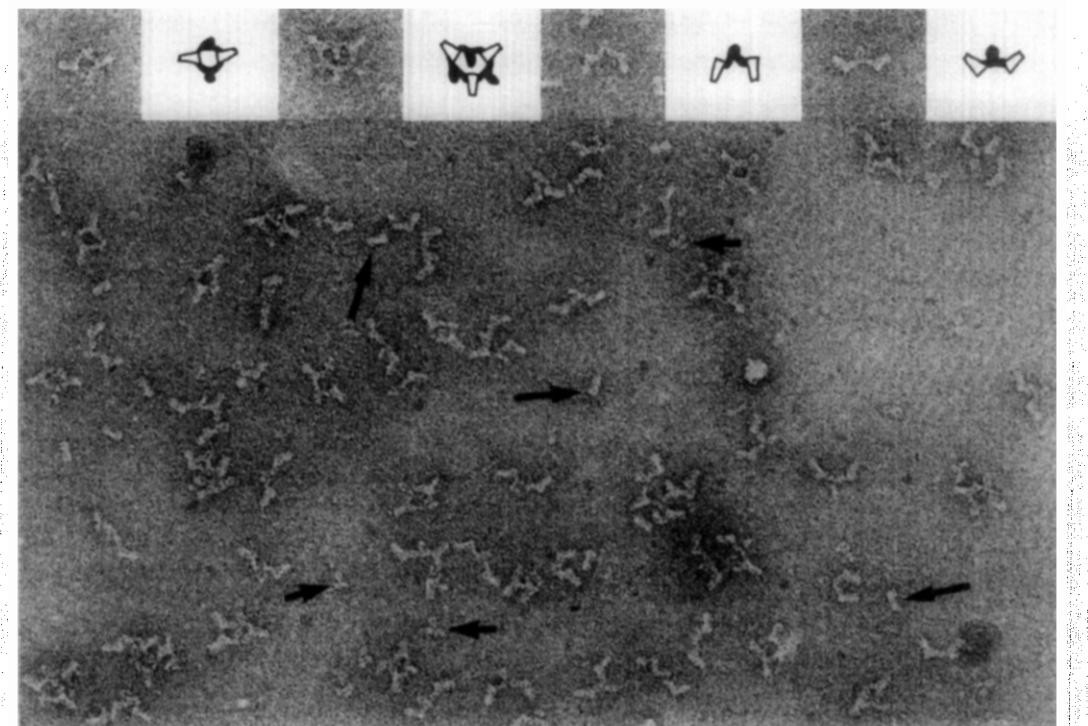


FIG. 1. Complexes of BHA with antibody HC3. For sealing, note free BHA molecules (long arrows), and free IgG molecules (short arrows). Magnification $\times 200,000$. To aid interpretation, the frames in Figs. 1-4 show four complexes with a sketch of each at right; BHA molecules are drawn open and IgG filled.

in PBS (pH 7.2) to 50 $\mu\text{g}/\text{ml}$ for optimum spreading on carbon support films. Antibody was added in increasing amounts until only a few molecules remained unbound to BHA after about 5 min equilibration. BHA-antibody complex suspensions were adsorbed to thin carbon films freshly stripped from mica, floated on a 1% w/v solution of sodium silicotungstate (pH 7.0) and then air dried. Micrographs were taken under minimum dose conditions known from analyses of periodic specimens to preserve detail below 1.5 nm; defocus was such that phase contrast was transferred in a continuous band down to about 1.0 nm (Wrigley *et al.*, 1983).

RESULTS

The information obtained by electron microscopy is presented in Fig. 1-4, each showing BHA complexed with a different antibody. (a) Figure 1: Antibody HC3 recognizes amino acid 144 in antigenic site A.

This antibody binds near an end of BHA, each Fab region forming a constant angle of about 110° with the BHA. Complexes of 1 + 1, 2 + 2, 2 + 1, 1 + 2, 3 + 3, etc. of BHA and antibody are readily seen, with the ring form of 2 + 2 most easily distinguished. (b) Figure 2: Antibody HC19 recognizes amino acid 157 in antigenic site B. In the BHA-antibody complexes shown the antibody binds to an end of the BHA at a constant angle of about 140°. As in Fig. 1, 1 + 1, 1 + 2, 2 + 2, 2 + 1, BHA-antibody complexes are seen, though 3 + 3 and higher was rare. (c) Figure 3: Antibody HC31 recognizes amino acid 198 in a different region of antigenic site B from amino acid 157 noted above in (b). The antibody also binds to a distinct site from HC19 on an end of the BHA forming angles of about 180°. Presumably as a consequence of the position of antibody binding only 1 + 1 and 2 + 1 BHA-antibody complexes are seen. (d) Figure 4: Antibody HC45 recognizes amino acid 63 in site E and binds near but at a site clearly removed from an

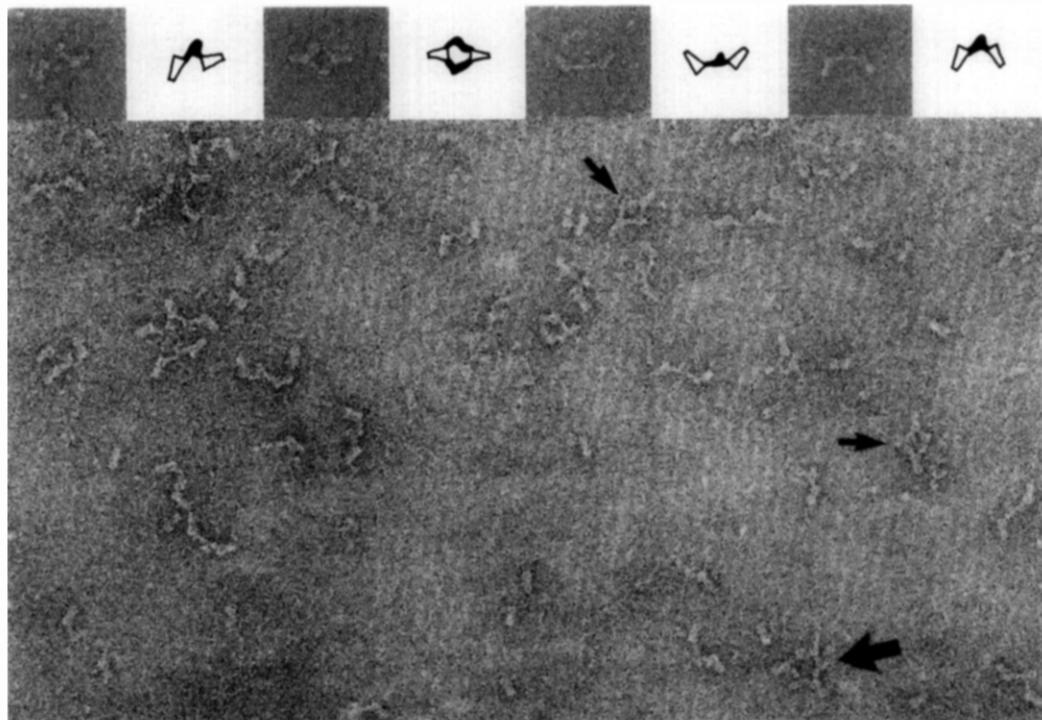


FIG. 2. Complexes of BHA with antibody HC19. Closed 2 + 2 and 3 + 3 rings arrowed. Magnification $\times 200,000$.

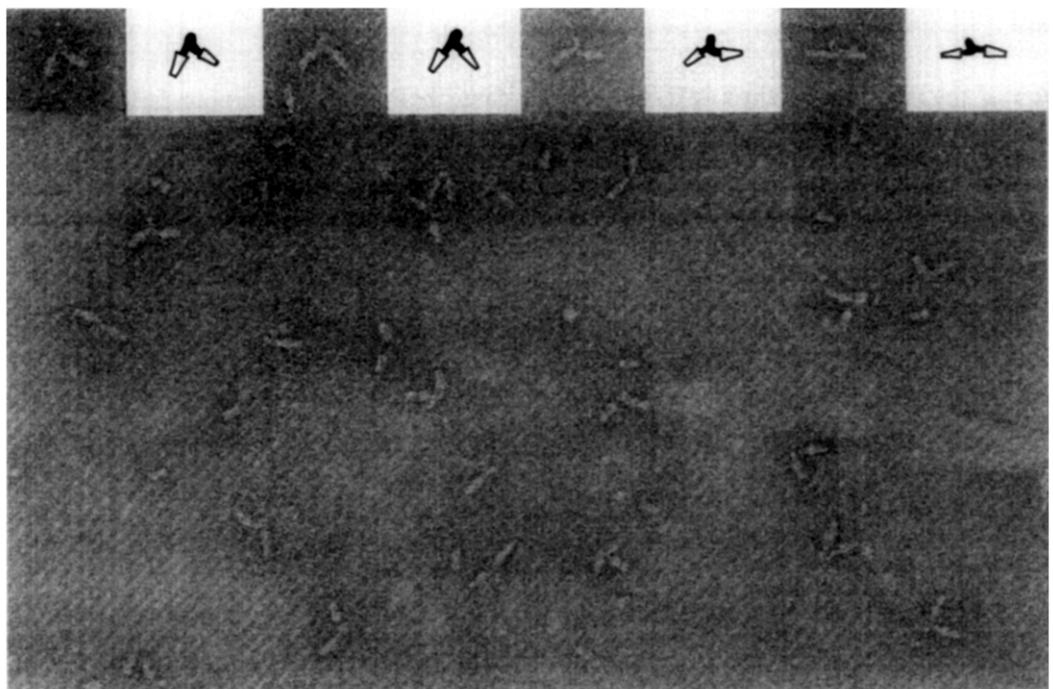


FIG. 3. Complexes of BHA with antibody HC31. Note the in-line bonding of Fab segments with the BHA long axis, and the absence of any complexes larger than 2 BHA + 1 antibody. Magnification $\times 200,000$.

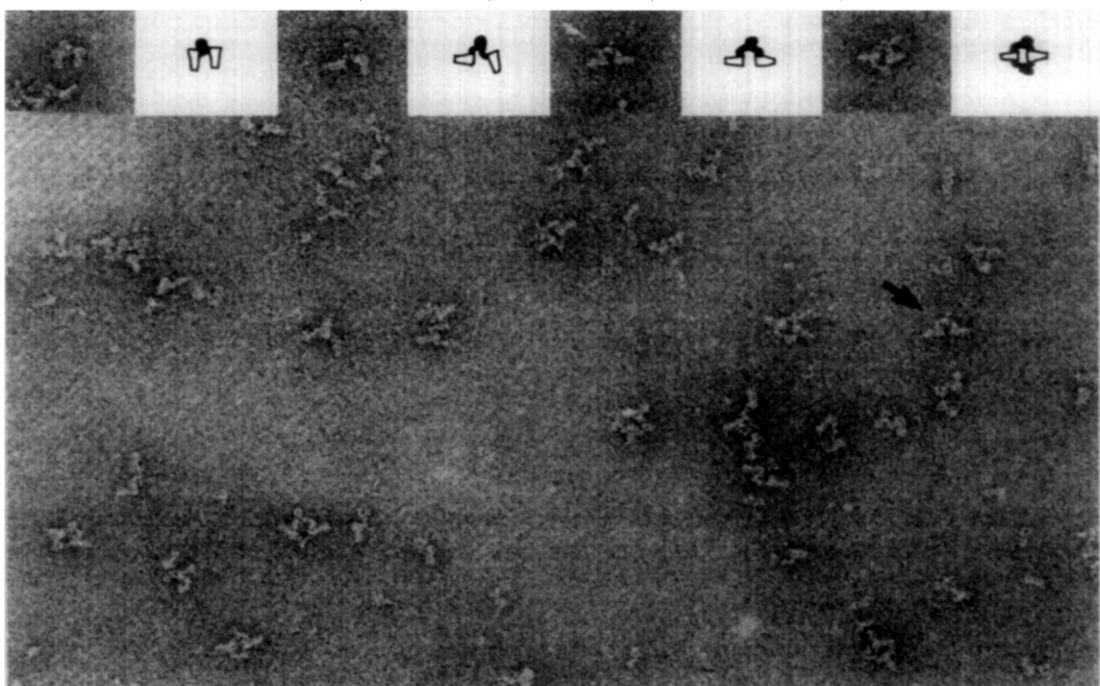


FIG. 4. Complexes of BHA with antibody HC45. In the closed 2 + 2 complexes (see also Fig. 5c, top row) note the apparent Fab bending to accommodate the 90° bond angle, shown also in 2 + 1 (arrow). Magnification $\times 200,000$.

end of the BHA. Again the angle subtended by the Fab of the bound antibody is constant and in this case 90°. Complexes of 1 + 1, 1 + 2, 2 + 2, etc., are readily seen.

Complexes formed between BHA and mixtures of monoclonal antibodies. All four antibodies were also mixed with BIIA two at a time. The six combinations (3/19, 3/31, 3/45, 19/31, 19/45, and 31/45) formed complexes which were mixtures of those

seen in Figs. 1–4 characteristic for each constituent antibody. In addition, most clearly in the 31/45 combination, but also in 31/3 and 31/19 combinations, complexes formed by both antibodies binding to single BHAs were also discerned, Fig. 5. The nature of these complexes (mainly 2 + 2 rings) indicates that all four antibodies bind to sites at the same end of the BHA. This conclusion can also be drawn from ex-

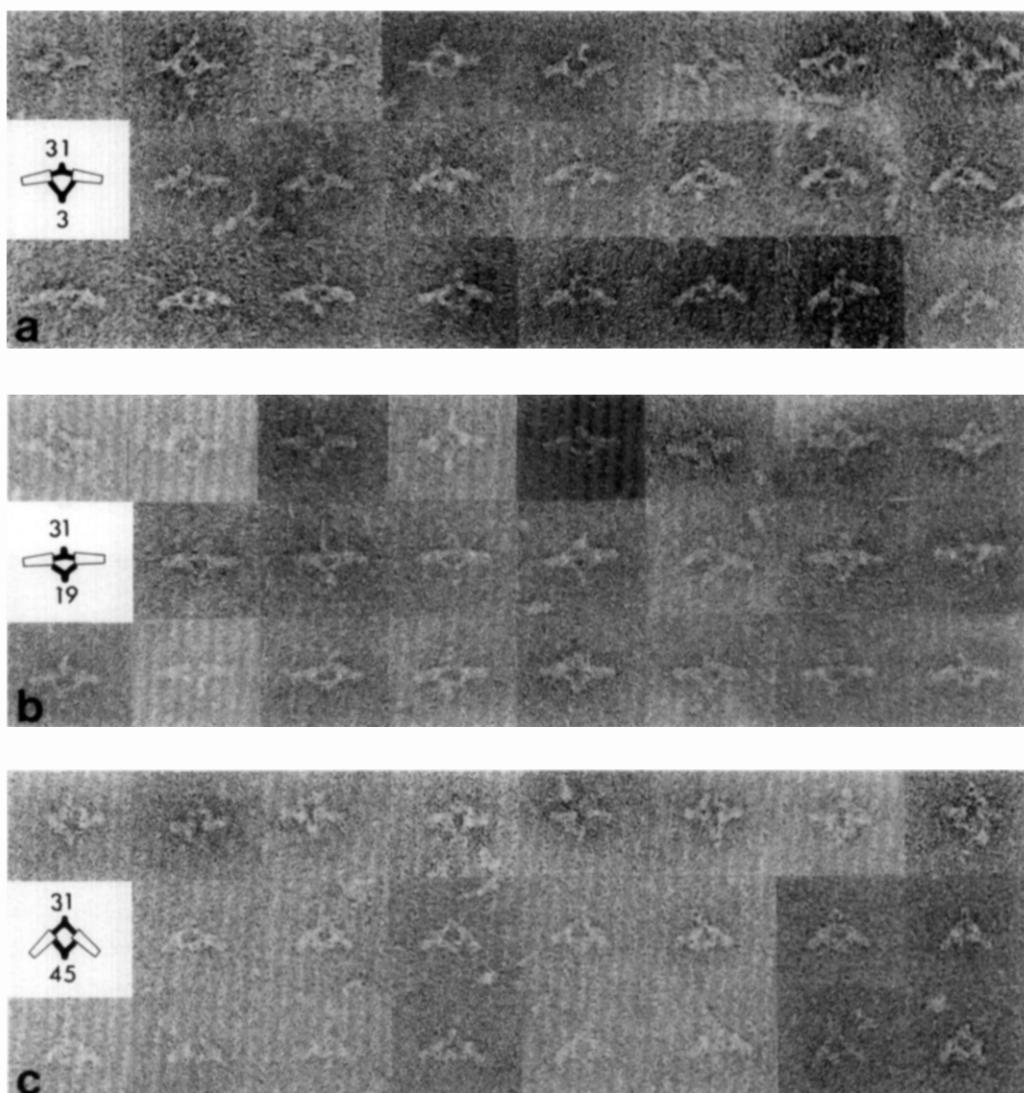


FIG. 5. 2 + 2 complexes of BHA with two different antibodies: (a) 31/3; (b) 31/19; (c) 31/45. HC31 is common to all as its binding (see Fig. 3) is easiest to distinguish from the others; it is placed uppermost in each complex as shown in the sketches. The top row of each panel shows control 2 + 2 complexes where both antibody molecules are the same: (a) HC3; (b) HC19; (c) HC45. Magnification $\times 250,000$.

amination of the other antibody combinations although the position and binding angle of HC3; HC19, and HC45 are less easy to distinguish from one another than from HC31.

DISCUSSION

These results directly demonstrate the sites of binding of four monoclonal antibodies of different specificities to the X-31 BHA. That the sites are different in detail for the different antibodies may be deduced from the observations that the angles formed between Fab regions and the BHA are constant and different for each antibody. The observation that these angles are constant irrespective of the type of complex observed, 1 + 1, 1 + 2, 2 + 2, BHA-antibody, etc., is allowed by the axial-rotation and flexing properties of the switch and hinge regions of the antibody molecules (Wrigley *et al.*, 1983).

Differences in the sites of antibody binding on the BHA can also be observed directly even at this level of resolution (probably about 2 nm). For example HC45 clearly binds at a site away from an end of the BHA molecule whereas HC31 binds squarely on an end. This location of the HC31 antibody-binding site is supported by the observation of restricted complex formation. Only 1 + 1 and 2 + 1 BHA-antibody complexes were seen (Fig. 3) presumably because of the binding of a second antibody to an unoccupied equivalent region of the BHA trimer is sterically prevented by the first antibody bound.

The sites of antibody binding and the locations of amino acid substitutions in the BHAs of antigenic variants can also be considered in relation to the three-dimensional structure of the BHA, Fig. 6. These considerations indicate that the sites of amino acid substitution are in all four cases appropriately located to be involved directly in the antibody-binding sites. In the case of HC31 they also indicate the basis of the steric inhibition of the binding of a second antibody to the BHA. Since the three 198 residues recognized are only 15 Å apart around the threefold axis of the trimer, the BHA is effectively monovalent

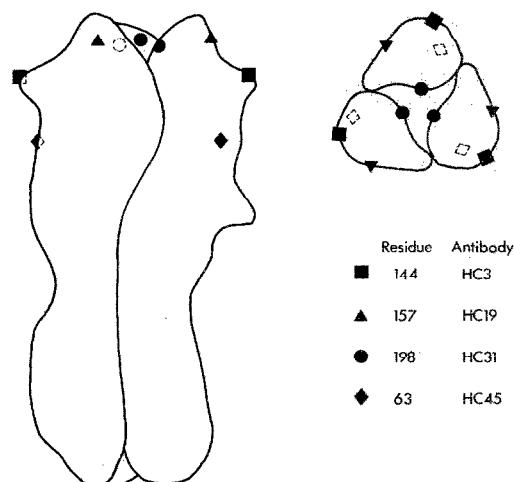


FIG. 6. Drawing of the BHA trimer showing the approximate sites of antibody binding and the locations of amino acid substitutions. Left: long axis vertical, distal end at the top. Right: view of the distal end; note the proximity of the three residues 198 to the trimer axis.

at this site. The results therefore substantiate the positions of antigenic sites A, B, and E and in the case of site B give an indication of the extent of this region at the distal tip of the BHA molecule. It would also be of interest to examine antibodies specific for sites C and D but we have not characterized antibodies prepared against X-31 virus which recognize amino acids in these sites.

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REFERENCES

- BOTH, G. W., and SLEIGH, M. J. (1981). Conservation and variation in the haemagglutinins of Hong Kong subtype influenza viruses during antigenic drift. *J. Virol.* **39**, 663–672.
- BRAND, C. M., and SKEHEL, J. J. (1972). Crystalline antigen from the influenza virus envelope. *Nature (London)* **238**, 145–147.
- CATON, A. J., BROWNLEE, G. G., YEWDELL, J. W., and GERHARD, W. (1982). The antigenic structure of the influenza virus A/PR/8/34 haemagglutinin (H1 subtype). *Cell* **31**, 417–427.

- COMPANS, R. W., and KLENK, H. D. (1979). In "Viral Membranes in Comprehensive Virology" (H. Fraenkel-Conrat and R. R. Wagner, eds.), pp. 293-407. Plenum, New York.
- DANIELS, R. S., DOUGLAS, A. R., SKEHEL, J. J., and WILEY, D. C. (1983). Analyses of the antigenicity of influenza haemagglutinin at the pH optimum for virus-mediated membrane fusion. *J. Gen. Virol.* **64**, 1657-1662.
- FAZEKAS DE ST. GROTH, S., and SCHEIDEGGER, D. (1980). Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Meth.* **35**, 1-21.
- GERHARD, W., YEWDELL, J., FRANKEL, M. E., and WEBSTER, R. (1981). Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. *Nature (London)* **290**, 713-717.
- JACKSON, D. C., MURRAY, J. M., WHITE, D. O., and GERHARD, W. U. (1982). Enumeration of antigenic sites of influenza virus haemagglutinin. *Infect. Immun.* **37**, 912-918.
- KILBOURNE, E. D. (1969). Future influenza vaccines and the use of genetic recombinants. *Bull. WHO* **41**, 643-645.
- KOHLER, G., and MILSTEIN, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (London)* **256**, 495-497.
- KOHLER, G., and MILSTEIN, C. (1976). Derivation of specific antibody-producing tissue culture and tumour lines by cell fusion. *Eur. J. Immunol.* **6**, 511-519.
- LAVER, W. G., AIR, G. M., WEBSTER, R. G., GERHARD, W., WARD, C. W., and DOPHEIDE, T. A. A. (1979). Antigenic drift in type A influenza virus: Sequence differences in the haemagglutinin of Hong Kong (H3N2) variants selected with monoclonal hybridoma antibodies. *Virology* **98**, 226-237.
- LAVER, W. G., AIR, G. M., DOPHEIDE, T. A., and WARD, C. W. (1980). Amino acid sequence changes in the haemagglutinin of A/Hong Kong (H3N2) influenza virus during the period 1968-1977. *Nature (London)* **283**, 454-457.
- PATEL, T. P., MILLICAN, T. A., BOSE, C. C., TITMAS, R. C., MOCK, G. A., and EATON, M. A. W. (1982). Improvements to solid phase phosphotriester synthesis of deoxyoligonucleotides. *Nucleic Acids Res.* **10**, 5605-5620.
- SANGER, F., NICKLEN, S., and COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
- SHULMAN, M., WILDE, C. D., and KOHLER, G. (1978). A better cell line for making hybridomas secreting specific antibodies. *Nature (London)* **276**, 269-270.
- VERHOEYEN, M., FANG, R., MIN JOU, W., DEVOS, R., HUYLEBROECK, D., SAMAN, E., and FIERS, W. (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.
- WARD, C. W. (1982). Structure of the influenza haemagglutinin. *Curr. Top. Microbiol. Immunol.* **94/95**, 1-74.
- WEBSTER, R. G., and LAVER, W. G. (1980). Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus haemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. *Virology* **104**, 139-148.
- WILEY, D. C., WILSON, I. A., and SKEHEL, J. J. (1981). Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature (London)* **289**, 373-378.
- WRIGLEY, N. G., BROWN, E., and CHILLINGWORTH, R. K. (1983). Combining accurate defocus with low-dose imaging in high resolution electron microscopy of biological material. *J. Microsc.* **130**, 225-232.
- WRIGLEY, N. G., BROWN, E. B., and SKEHEL, J. J. (1983). Electron microscope evidence for the axial rotation and inter-domain flexibility of the Fab regions of immunoglobulin G. *J. Mol. Biol.* **169**, 771-774.