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Morphology and antigenicity studies on reassortant influenza (H3N2) viruses for use in inactivated vaccines

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SUMMARY

Three influenza A (H3N2) reassortant whole virus vaccine strains with differing antibody-inducing capacities in hamsters were investigated morphologically and antigenically. Although initial measurements of virion circumference, from electron micrographs of vaccine preparations, suggested a relationship of small virion size with low immunogenicity, subsequent immunization with, and morphological investigation of, vaccine virions separated on sucrose gradients, failed to obtain populations whose antibody-inducing capacity clearly correlated with constituent virion density, size, morphology or integrity.

However, antigenic investigation using single radial haemolysis (SRH) and monoclonal antibodies revealed significant differences in antigenic specificity between the strains. Furthermore, a series of H3N2 isolates, derived using standard reassortment procedures, also showed differences in antigenic specificity in their haemagglutination-inhibition (HI) reactions with monoclonal antibodies after five passages in allantois-on-shell cultures. Variation between these isolates and their A/Victoria parent virus could be detected using SRH and hamster sera raised against each isolate.

These results demonstrate variation between candidate influenza A virus vaccine strains, all possessing the same surface (H3N2) glycoproteins, expressed as a consequence of the reassortant system used for their production.

INTRODUCTION

Traditionally, strategies for immunization against influenza A viruses have emphasised the importance of inducing protective antibody to the viral surface components (Hobson, Curry & Beare, 1973; Smith & Davies, 1977; Oxford *et al.* 1979). Selection of appropriate reassortant strains having good growth capacity in eggs and possessing the same surface haemagglutinin (HA) and neuraminidase (NA) antigens as the currently circulating strain has involved the use of mixed infections under varying *in vitro* conditions (Haaheim & Schild, 1976).

However, numerous epidemiological studies document the frequent occurrence of drifted HA (Yewdell, Webster & Gerhard, 1979) and NA (Laver *et al.* 1982) antigens as determined by nucleotide sequencing (Verhoeyen *et al.* 1980), peptide mapping (Laver & Webster, 1968) and reactions with monoclonal antibodies (Webster & Laver, 1980) in naturally occurring influenza viruses. Studies with both

influenza A and influenza B viruses (Brand & Palese, 1980; Webster & Berton, 1981; Gitelman *et al.* 1983), have shown such antigenic variants to be manifest *in vitro* as a product of multiple passaging in animals, eggs or tissue culture. Recent work by Schild *et al.* (1983) with influenza B virus has shown that differential host cell tropisms of virus subpopulations can lead to selection of antigenically distinct clones which, according to their passage history after isolation, can be defined by reactions with a monoclonal antibody panel.

Previous studies in this laboratory (Hamzawi, Jennings & Potter, 1981) have shown that a series of ten reassortant influenza A viruses, each possessing surface antigens derived from their H3N2 A/Victoria/75 parent strain, will apparently induce antibody of differing HI titre in hamsters when inoculated at similar dosage levels.

Following observations on the size and morphological characteristics of three of these influenza A viruses, a 'high' (VFEB82), 'intermediate' (A/Victoria/75) and 'low' (VP1) antibody inducer, we have investigated the possibility of a morphological basis for the different immunogenicities observed, looked for evidence of antigenic variation among these viruses, and also for evidence of antigenic variation occurring within the reassortant system used for their production and propagation.

Findings indicate that the basis for the differing immunogenicities of the reassortant strains lies in an altered antigenic specificity of the viral haemagglutinin. Implications for vaccine production are discussed.

MATERIALS AND METHODS

Virus strains and vaccine preparations

The source of A/Victoria/3/75 (H3N2) and A/PR/8/34 (H1N1) viruses, and the derivation of the reassortant influenza viruses VFEB82 (A/FM/1/47 × A/Victoria/75), VP1 and RIT 4050 (A/PR/8/34 × A/Victoria/75), together with their propagation and cloning, and the preparation of vaccines from them, have been previously described (Hamzawi, Jennings & Potter, 1981).

Animals

Eight to 12-week-old randomly bred Syrian hamsters were obtained from a closed colony at the University of Sheffield.

Monoclonal antibodies

Four monoclonal antibodies (as mouse ascitic fluids), HC59, HC14 and HC20, raised against A/Port Chalmers/1/73 HA and HC170, raised against A/England/864/75 HA were a gift from Dr A. Douglas, World Influenza Centre, NIMR, Mill Hill, London.

Single radial diffusion

The method of Wood *et al.* (1977) was used to determine the HA concentration of inactivated vaccine preparations. Standard A/Victoria antigen was obtained from the National Institute for Biological Standardisation and Control, Holly Hill, London.

Physical characteristics of virions in inactivated vaccines

(*a*) *Virion size and morphology.* After adjustment of vaccine concentrations to 40 μg HA/ml, samples were prepared for electron microscopy using, essentially, the method of Norang & Codd (1979).

Virus particle circumference was measured from representative electron-micrographs using an M.O.P 2 (Manual Optional Picture, Model 2, Reichert-Jung).

(*b*) *Separation of virions in density gradients.* Concentrated, inactivated virus vaccine preparations were layered onto 5–40 % linear sucrose density gradients. After ultracentrifugation for 20 min at 20000 ***g***, 12 or 13 fractions were collected, their HA and protein content estimated, and fractions from two areas of each gradient pooled, to give dense and light particle preparations. Final purification involved ultracentrifugation at 40000 ***g*** for 90 min.

Antiserum preparation

Each vaccine preparation in 0·5 ml amounts containing 20 μg HA was administered to hamsters intramuscularly. Sera were obtained following cardiac puncture 21 days later.

Serological tests

(*a*) *Haemagglutination-inhibition* (*HI*). Sera were tested in microplates using four haemagglutinating units (HAU) of antigen as described previously (Hamzawi, Jennings & Potter, 1981) except that sera were pre-treated overnight using potassium periodate (Hirst, 1948).

(*b*) *Single radial haemolysis* (*SRH*). The method was essentially that of Oxford *et al.* (1979), with modifications described elsewhere (Goodeve, Jennings & Potter, 1983; Jennings *et al.* 1984). Between sera tested on the same immunoplate, differences in zone area of $\geqslant 48\,\%$ were significant and this agrees well with values quoted elsewhere for influenza A viruses (Oxford, Yetts & Schild, 1982; Jennings *et al.* 1984; Al-Khayatt, Jennings & Potter, 1984). Standard reference antisera were used to eliminate interplate variations.

Homology indices were used for the assessment of antigenic relatedness between viruses, determined according to a method described by Russell, McCahon & Beare (1975), where

$$\text{homology index} = \frac{\text{zone area when tested } vs \text{ heterologous virus strain}}{\text{zone area when tested } vs \text{ homologous virus}}.$$

The figure for intraplate variation, $\geqslant 48\,\%$ (1·48 fold), was used as a basis for assessing specificity. Therefore to account for maximum possible variation, values of 1·48 or 0·68 (1/1·48) were used to derive a homology index value of 1.48/0·68 = 2·18. Homology indices of $\geqslant 2{\cdot}18$ or $\leqslant 0{\cdot}48$ (1/2·18) were subsequently used to indicate significant differences in specificity.

Preparation of cloned passaged virus from mixed infections

Ten-day embryonated hens' eggs were co-infected with cloned, parental influenza viruses A/Victoria and A/PR8. Infection, limit dilution passage in the presence of A/PR8 antiserum, incubation and passage in allantois-on-shell (AOS) cultures have all been described (Hamzawi, Jennings & Potter, 1981).

Table 1. *Size and form of virions from unfractionated and fractionated reassortant and parental influenza virus vaccines*

	Unfractionated vaccine		Sucrose gradient fractionated vaccine preparations				
Concentrated virus vaccine preparation	Virion circumference < 400 nm	Mean virion circumference	Fraction	Virion circumference < 400 nm	Mean virion circumference	Incidence of intact virions	Incidence of spherical virions
VFFB82	254/384 (66)*	434	Light (9–14·0% sucrose)	45/75 (60)	424	103/110 (94)	14/110 (13)
			Dense (38–40% sucrose)	72/83 (87)	209	26/51 (51)	17/51 (33)
A/Victoria/75	239/447 (54)	447	Light (5–9% sucrose)	63/101 (62)	443	67/90 (74)	9/90 (10)
			Dense (38–40% sucrose)	54/78 (69)	379	28/73 (38)	29/73 (40)
VP1	356/431 (83)	329	Light (6–12% sucrose)	120/132 (91)	297	84/135 (62)	58/135 (43)
			Dense (38–40% sucrose)	48/74 (65)	430	28/39 (72)	12/39 (31)

* Number of virions with size or form shown /Total number of particles assessed (%).

Table 2. *Serum HI and SRH antibody responses of hamsters to light and dense fractions of influenza virus vaccine preparations*

Hamster sera raised using:		Serum HI antibody responses × RIT 4050		SRH antibody responses × RIT 4050	
Virus vaccine strain	Gradient fraction	Number of sera positive/ total tested	Mean titre*	Number of sera positive/ total tested	Mean titre
VFEB82	Light/top (9–14 % sucrose)	7/7	58·0	7/7	79·8
	Dense/bottom (38–40 % sucrose)	8/8	98·9	8/8	176·9
A/Victoria/75	Light/top (5–9 % sucrose)	10/10	63·5	10/10	36·3
	Dense/bottom (38–40 % sucrose)	13/16	19·5†	13/16	36·3
VPI	Light/top (6–12 % sucrose)	6/10	22·1	6/10	< 20·0†
	Dense/bottom (38–40 % sucrose)	13/17	13·2†	13/17	27·8†

* Mean values of SRH tests expressed as zone areas (mm^2); mean HI titres expressed as reciprocal geometric mean values.

† Significantly different from HI or SRH titres induced by vaccine prepared from the dense fraction of VFEB82.

HI and neuraminidase-inhibition (NI) tests (Hamzawi, Jennings & Potter, 1981) were used to identify strains bearing H3N2 surface antigens after two limit dilution passages in the presence of A/PR8 antiserum. Allantoic fluid pools from five cloned isolates at this stage, and also following a further three passages in AOS cultures were prepared in whole eggs.

RESULTS

Physical characteristics of vaccines prepared from reassortant and parental H3N2 strains

To determine a possible association between morphological characteristics of VP1, VFEB82 and A/Victoria, and their differing antibody-inducing capacity, various properties of the strains were investigated. Measurements of virion circumference show (Table 1) that vaccine prepared from VP1 contained significantly more particles of smaller size than that prepared from either A/Victoria ($\chi^2 = 85{\cdot}3$; $P = < 0{\cdot}001$) or VFEB 82 ($\chi^2 = 29{\cdot}2$; $P = < 0{\cdot}001$). Indeed, only 17 % of virions in the VP1 preparation had circumferences of > 400 nm.

Table 1 also compares virion size, as determined from circumference measurements, in preparations from both light (5–14 % sucrose) and dense (38–40 % sucrose) regions of sucrose gradients for each strain. There was no overall correlation of virions of small circumference (< 400 nm) with either dense or light fraction preparations, and although vaccine prepared from the dense fraction of VFEB82

contained significantly smaller particles than vaccine prepared from the light fraction of the same virus strain, ($\chi^2 = 14{\cdot}7$; $P = < 0{\cdot}001$), the situation was reversed for VP1 ($\chi^2 = 21{\cdot}4$; $P = < 0{\cdot}001$). In fact, vaccines prepared from the dense fraction of VFEB82 and the light fraction of VP1 both contained significantly greater numbers of particles of virion circumference < 400 nm, with one exception, than all other vaccine preparations.

From electron micrographs of pooled fractions from dense and light regions of the gradient for each virus strain, estimates of numbers of intact as opposed to disrupted, and of spherical as opposed to non-spherical particles gave no correlation of particle integrity or particle shape with density (Table 1).

Antibody responses of hamsters to fractionated vaccine preparations

Immune sera from hamsters inoculated with equivalent μg HA of each light and dense gradient fraction, for each vaccine, were tested by both HI and SRH using RIT 4050 as antigen.

With one exception, vaccines prepared from either the dense or light fractions of VFEB82 apparently induced higher antibody titres than all other preparations (Table 2). The small numbers of animals tested per group may have resulted in the rather poor correlation observed between HI and SRH antibody measurements.

To investigate further the basis for the qualitative difference in amounts of HI and SRH antibody induced by the three viruses, SRH tests were carried out in which sera from hamsters immunized with various unfractionated virus vaccines, were tested for antibody using sheep erythrocytes (SRBC) sensitized with equivalent HAU amounts of either homologous or heterologous virus. Table 3 shows that, under these conditions, high antibody-inducing capacity was not consistently related to VFEB82 vaccine. Thus, although the largest SRH zones were observed in sera raised using VFEB82 vaccine when homologous virus, or A/Victoria, were used as antigens, this was not so when these sera were tested using SRBC sensitized with VP1.

In addition, significant ($\geqslant 48\%$) differences in zone areas of haemolysis are apparent between VFEB82, A/Victoria or VP1 antisera tested on immunoplates containing any one of these virus strains as antigens (Table 3). The homology indices also indicate antigenic unrelatedness between the strains. Values of 2·26 for A/Victoria with VFEB82 antisera, 0·09 and 0·07 for VP1 with A/Victoria, and VFEB antisera respectively, and 0·32 for VFEB82 with VP1 antisera, all represent significant differences in antigenic specificity. These findings are supported by the results obtained when each vaccine preparation was tested by HI against four monoclonals (Table 3). Significant, $\geqslant$ 4-fold, differences between VP1 and the other two strains were revealed using monoclonals HC59 and HC14.

Reactions of cloned passaged virus with monoclonal antibodies

The parental and reassortant strains studied above were the product of the double-infection system for making reassortant viruses, and all strains had undergone at least 12 passages in either AOS cultures and eggs (VFEB82 and A/Victoria) or in tissue cultures and eggs (VP1), before investigation for antigenic differences.

Table 3. *Antigenic differences between reassortant and parental H3N2 influenza viruses detected by both SRH and HI using immune hamster sera or monoclonal antibodies*

	SRH test						HI test: Mouse monoclonal antibody no.			
	Hamster antisera × VFEB82		Hamster antisera × A/Victoria/75		Hamster antisera × VP1					
Virus strain used as antigen	Mean ± S.D. (Range)	Homology Index	Mean ± S.D. (Range)	Homology Index	Mean ± S.D. (Range)	Homology Index	HC170	HC59	HC14	HC20
VFEB82	52·0 ± 25* (18·5–109·8)	1·00	1·2 ± 3·7 (0·5–11·8)	0·07	13·3 ± 11·5 (0·5–29·3)	0·32	160†	⩾ 40960	10240	10240
A/Vic/75	117·8 ± 36 (76·3–199·1)	2·26	16·6 ± 14·2 (0·5–44·5)	1·00	51·0 ± 27·2 (13·4–91·5)	1·23	160	⩾ 40960	10240	1280
VP1	29·1 ± 25·6 (0·5–76·3)	0·56	1·5 ± 4·7 (0·5–15·0)	0·09	41·5 ± 16·6 (6·8–60·1)	1·00	160	5120	⩾ 40960	160

S.D., standard deviation.

$$\text{Homology index} = \frac{\text{zone area when tested } vs \text{ heterologous virus strain}}{\text{zone area when tested } vs \text{ homologous virus}}.$$

Ratio of 1.00 indicates absolute homology.

* SRH values expressed as zone areas (mm^2).

† HI titres expressed as the reciprocal of the highest antibody dilution causing complete inhibition of 4HAU of virus.

Table 4. *HI reactivities of five cloned H3N2 mixed infection isolates with monoclonal antibodies after two and five passages in allantois-on-shell cultures*

Virus clone used as antigen	Passage level in A-O-S cultures	Monoclonal antibody number			
		HC170	HC59	HC14	HC20
A	2	120*	900	800	900
B	2	100	3400	500	1700
C	2	80	1100	700	2800
D	2	100	1500	900	1500
E	2	100	1600	900	1800
A	5	130	17700	6900	5300
B	5	140	4800	4000	8600
C	5	130	5100	2200	8600
D	5	120	8600	3000	35000
E	5	180	53900	3600	76200
A/Victoria/75	—	70	2900	1500	3200

* Titres expressed as the reciprocal of the highest serum dilution causing complete inhibition of 4HAU of virus. The values represent the geometric means of four repeat experiments.

Using A/PR8 and A/Victoria as parental viruses, a further series of cloned virus isolates, all bearing parental H3N2 surface antigens, and all derived by similar procedures, were used to assess the effect of sequential passage on their antigenic specificity. Table 4 shows the HI reactions, with four monoclonal antibodies, of allantoic fluid pools prepared from five cloned isolates after either two or five passages in AOS cultures. After two cloned passages, all clones and their A/Victoria parent showed similar reactivities ($\leqslant$ 4-fold differences) with each monoclonal. However, after five passages in AOS cultures clone E showed $\geqslant$ 8-fold differences in HI titre from clones A, B, C and A/Victoria in reactivity with monoclonal HC20, and $\geqslant$ 6-fold differences from clones B, C, D and A/Victoria with monoclonal HC59. Significant differences were also seen between clone D, and clones A, B, and C and A/Victoria using monoclonal HC20, and between clone A and A/Victoria using HC59 and HC14.

Reactions of antisera to cloned passaged virus with A/Victoria/75 by SRH.

To further assess the antigenic relatedness of the cloned isolates with respect to their A/Victoria parent, antisera against each clone, at passage levels two and five, were prepared in hamsters and tested by SRH, together with hamster antisera against A/Victoria, using A/Victoria as antigen (Table 5).

At passage two, hamster antisera against each of the clones showed similar SRH reactions with A/Victoria virus. However, these zones of haemolysis were markedly greater than those observed in the reaction of homologous antisera with A/Victoria. This altered immunogenicity after two passages suggests a similar antigenic modification in all the clones, possibly resulting from the presence of A/PR8 antiserum present during the first two cloned passages but not subsequently included.

At passage level five however, antisera to clone A produced SRH zone areas,

Table 5. *SRH reactivity with A/Victoria/75 virus of hamster antisera to five cloned H3N2 isolates after two and five passages in allantois-on-shell cultures*

Hamster antiserum to clone number	Passage level in A-O-S cultures	SRH results against A/Victoria/75 Antigen		
		No. of sera tested	Range of zone area	Mean ± S.D. zone area
A	2	7	*52·4–111·7	75·6 ± 22
B	2	8	13·4–88·0	63·6 ± 24
C	2	8	26·1–95·0	61·1 ± 31
D	2	8	22·2–109·9	66·1 ± 29
E	2	6	39·5–129·8	81·3 ± 35
A	5	8	17·6–39·5	25·9 ± 7
B	5	7	40·7–73·1	55·0 ± 14
C	5	8	23·1–53·8	35·7 ± 9
D	5	8	12·6–60·1	33·5 ± 17
E	5	4	14·2–45·7	36·5 ± 13
A/Victoria/75 (H3N2 parental virus)	—	5	17·6–56·6	36·5 ± 16

* SRH Values expressed as zone areas (mm^2).

in reaction with A/Victoria antigen, that were significantly different from those elicited by clones B and D, while antisera to clone B induced zone areas significantly different from those of clones C and E. There was also a significant difference in the zone areas produced by antisera to clone A at passage level five, and those seen with antisera to A/Victoria.

DISCUSSION

Previous work in this laboratory described a series of reassortant, H3N2, influenza A viruses which when inoculated as inactivated whole virus vaccines into hamsters at equivalent dosages, apparently differed in their ability to induce serum HI antibody (Hamzawi, Jennings & Potter, 1981). In the case of 'low' antibody inducer, VP1, the present studies suggest a correlation of small virion size with low immunogenicity. Both reassortants possessed matrix protein derived from the non-A/Victoria parent virus (Hamzawi, Jennings & Potter, 1981), and association of a 'foreign' matrix protein with A/Victoria surface proteins during maturation could result in virions with physical or biological properties altered in some respect relevant to their immunogenicity. However, density gradient profiles (results not shown) of VFEB82 and VP1 indicated similar particle distribution, and no individual population within the three strains examined exhibited an immunogenicity correlated exclusively with virion density, size, morphology or integrity. Subpopulations of wild-type influenza A viruses have been described where varying matrix protein content was associated with altered buoyant density and particle morphology (Kendal, Galphin & Palmer, 1977), although these virus strains were not studied for variation in antibody-inducing capacity.

These results led us to investigate more closely the antigenic relatedness of the

reassortants and A/Victoria. Recent studies have shown that passage of influenza viruses in animals, eggs or tissue culture can produce variants in antigenicity detectable by oligonucleotide mapping, RNA hybridization analysis, or monoclonal antibody (Brand & Palese, 1980; Gitelman *et al.* 1983; Naeve, Webster & Hinshaw, 1983; Downie, 1984). Both reassortants used in the present studies had received at least 12 serial passages from their derivation which may have led to the antigenic variation between them and A/Victoria virus demonstrable using SRH and monoclonal antibodies.

In deriving reassortants with the necessary growth capacity (Kilbourne *et al.* 1971), and preparing these strains for use in inactivated influenza virus vaccines, at least five passages are required, and using monoclonal antibodies and SRH tests, the present studies show detectable variation between five H3N2 antigen-bearing cloned isolates and A/Victoria after just five passages in AOS cultures.

Downie (1984) has recently stressed the importance of using discriminating tests to characterize influenza strains derived by standard reassortant procedures for use in vaccines and recommends that both RNA hybridization analysis and monoclonal antibodies be employed to ensure the antigenic relatedness of the virus strain finally selected corresponds closely to that of the wild-type virus against which protection is desired. Our studies are in agreement with this, and show that both the SRH test and a panel of monoclones are sufficiently sensitive to distinguish differences between closely-related strains following the five or more serial passages needed to produce an influenza virus vaccine.

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REFERENCES

AL-KHAYATT, R., JENNINGS, R. & POTTER, C. W. (1984). Interpretation of responses and protective levels of antibody against attenuated influenza A viruses using single radial haemolysis. *Journal of Hygiene* **93**, 301–312.

BRAND, C. & PALESE, P. (1980). Sequential passage of influenza virus in embryonated eggs or tissue culture: Emergence of mutants. *Virology* **107**, 424–433.

DOWNIE, J. C. (1984). A genetic and monoclonal analysis of high-yielding reassortants of influenza A viruses used for human vaccines. *Journal of Biological Standardization* **12**, 101–110.

GITELMAN, A. K., KAVERIN, N. V., KHARITONENKOV, I. G., RUDNEVA, I. A. & ZHDANOV, V. M. (1983). Antigenic changes in mouse-adapted influenza virus strains. *Lancet* i, 1229.

GOODEVE, A. C., JENNINGS, R. & POTTER, C. W. (1983). The use of the single radial haemolysis test for assessing antibody response and protective antibody levels in an influenza B vaccine study. *Journal of Biological Standardization* **11**, 289–296.

HAAHEIM, L. E. & SCHILD, G. C. (1976). Antigenic variants of influenza A virus obtained in vitro. *Bulletin of the World Health Organisation* **53**, 305–311.

HAMZAWI, M., JENNINGS, R. & POTTER, C. W. (1981). Antigenicity in hamsters of inactivated vaccines prepared from recombinant influenza viruses. *Journal of Hygiene* **87**, 453–464.

HIRST, G. K. (1948). Nature of virus receptors of red cells: evidence on chemical nature of virus receptors of red cells and existence of closely analogous substance in normal serum. *Journal of Experimental Medicine* **87**, 301–314.

HOBSON, D., CURRY, R. C. & BEARE, A. S. (1973). Haemagglutination-inhibition antibody titres as a measure of protection against influenza in man. *Developments in Biological Standardisation* **20**, 164–168.

JENNINGS, R., SMITH, T. L., SPENCER, R. C., MELLERSH, A. R., EDEY, D., FENTON, P. & POTTER, C. W. (1984). Inactivated influenza virus vaccines in man: a comparative study of subunit and split vaccines using two methods for assessment of antibody responses. *Vaccine* **2**, 75–80.

KENDAL, A. P., GALPHIN, J. C. & PALMER, E. L. (1977). Replication of influenza virus at elevated temperatures: Production of virus-like particles with reduced matrix protein content. *Virology* **76**, 186–195.

KILBOURNE, E. D., SCHULMAN, J. L., SCHILD, G. C., SCHLOER, G., SWANSON, J. & BUCHER, D. (1971). Correlated studies of a recombinant influenza virus vaccine. *Journal of Infectious Diseases* **124**, 449–462.

LAVER, W. G., AIR, G. M., WEBSTER, R. G. & MARKOFF, L. J. (1982). Amino acid sequence changes in antigenic variants of type A influenza virus N2 neuraminidase. *Virology* **122**, 450–460.

LAVER, W. G. & WEBSTER, R. G. (1968). Selection of antigenic variants of influenza viruses. Isolation and peptide mapping of their haemagglutinating proteins. *Virology* **34**, 193–202.

NAEVE, C. W., WEBSTER, R. G. & HINSHAW, V. S. (1983). Phenotypic variation in influenza virus reassortants with identical gene constellations. *Virology* **128**, 331–340.

NORANG, H. K. & CODD, A. A. (1979). A low-speed centrifugation technique for the preparation of grids for direct virus examination by electron microscopy. *Journal of Clinical Pathology* **32**, 304–305.

OXFORD, J. S., SCHILD, G. C., POTTER, C. W. & JENNINGS, R. (1979). The specificity of the anti-haemagglutinin antibody response induced in man by inactivated influenza vaccines and by natural infection. *Journal of Hygiene* **82**, 51–61.

OXFORD, J. S., YETTS, R. & SCHILD, G. C. (1982). Quantitation and analysis of the specificity of post-immunization antibodies to influenza B using single radial haemolysis. *Journal of Hygiene* **88**, 325–333.

RUSSELL, S. M., MCCAHON, D. & BEARE, A. S. (1975). A single radial haemolysis technique for the measurement of influenza antibody. *Journal of General Virology* **27**, 1–10.

SCHILD, G. C., OXFORD, J. S., DE JONG, J. C. & WEBSTER, R. G. (1983). Evidence for host cell selection of influenza virus antigenic variants. *Nature, London* **303**, 706–709.

SMITH, A. J. & DAVIES, J. R. (1977). The response to inactivated influenza A (H3N2) vaccines: the development and effect of antibodies to the surface antigens. *Journal of Hygiene* **78**, 363–375.

VERHOEYEN, M., FANG, R., MIN JOU, W., DEVOS, R., HUYLEBROECK, D., SAMAN, E. & 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.

WEBSTER, R. G. & LAVER, W. G. (1980). Determination of the number of non-overlapping antigenic areas of Hong-Kong (H3N2) influenza virus haemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. *Virology* **104**, 139–148.

WEBSTER, R. G. & BERTON, M. T. (1981). Analysis of antigenic drift in the haemagglutinin molecule of influenza B virus with monoclonal antibodies. *Journal of General Virology* **54**, 243–251.

WOOD, J. M., SCHILD, G. C., NEWMAN, R. W. & VALERIE, SEAGROATT (1977). Application of an improved single-radial immunodiffusion technique for the assay of haemagglutinin antigen content of whole virus and subunit influenza vaccines. In *Developments in Biological Standardisation*, vol. 39, pp. 193–200. Basel: S. Karger.

YEWDELL, J. W., WEBSTER, R. G. & GERHARD, W. (1979). Antigenic variation in three distinct determinants of an influenza A haemagglutinin molecule. *Nature, London* **279**, 246–248.