Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950

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Comparison of the oligonucleotide maps of the RNAs of current human influenza (H1N1) virus isolates shows these strains to be much more closely related to viruses isolated in 1950 than to strains which circulated before or after that period.

IN May 1977 influenza viruses of the H1N1 (haemagglutinin and neuraminidase) serotype were isolated in China[1]. Subsequently, additional H1N1 virus isolates were obtained in Russia and in the winter of 1977–78 epidemic infection with these H1N1 ('Russian') viruses was evident all over the Northern Hemisphere, including the United States[2,3]. Although investigators had speculated that the H3N2 subtype which first appeared in 1968 was approaching the end of its period of prevalence, the emergence of H1N1 virus was unexpected. H1N1 viruses were epidemic between 1946 and 1957 and a large proportion of the world's population over the age of 20 possesses serum antibodies against such viruses. Consequently, a strain with a novel haemagglutinin or one which had appeared less recently would have seemed a more likely candidate for the next pandemic strain. Because of the antigenic relatedness of the surface proteins of the 1946–57 H1N1 strains and the Russian isolates we set out to compare the genomes of these viruses by oligonucleotide mapping. Such

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Table 1 Summary of the comparison of oligonucleotide patterns derived from the RNAs of different influenza viruses

Strain	Spots missing*	Spots additional*	Total no. of spots evaluated†
A/FW/1/50 (H1N1)	—		44
A/USSR/90/77(H1N1)	3, 7, 26, 29, 35, 40	100, 101, 102, 103	42
A/USSR/92/77(H1N1)	3, 5, 7, 10, 12, 15, 26, 29, 30, 35, 40	100, 101, 102, 103, 104, 105	39
A/HK/123/77(H1N1)	5, 6, 7, 15, 16, 26, 29, 35, 40	101, 102, 103, 104, 105, 106, 107	42
A/I/2/50(H1N1)	5, 15, 26, 40	107, 108	42
A/C/1/56(H1N1)	1, 4, 6, 7, 8, 10, 11, 12, 15, 19, 21, 23, 24, 27, 29, 31, 33, 35, 39, 40	102, 103, 105, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120	37
A/FM/1/47(H1N1)	2, 3, 5, 6, 7, 10, 11, 12, 13, 15, 16, 19, 20, 23, 24, 25, 34, 35, 39, 40, 43	104, 105, 109, 113, 114, 118, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136	44
A/PR/8/35(H0N1)	2, 4, 5, 8, 9, 11, 12, 15, 16, 20, 22, 23, 24, 25, 27, 35, 40, 44	102, 106, 113, 114, 118, 119, 123, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147	45
A/PtCh/1/73(H3N2)	2, 3, 4, 7, 8, 9, 13, 15, 16, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 33, 35, 38, 40, 44	101, 102, 103, 113, 116, 127, 130, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166	46

The WHO system of nomenclature[24] is used here: this designates influenza viruses according to: type/place of isolation/isolate number/year of isolation (subtypes of haemagglutinin, H, and neuraminidase, N). The abbreviations FW, USSR, HK, I, C, FM, PR, PtCh refer to the following locations: Fort Warren, Wyoming; Soviet Union, place unknown; Hong Kong; Irvington House, New York; Cornell University, New York; Fort Monmouth, New Jersey; Puerto Rico; Port Chalmers, New Zealand.

* The ribonuclease T_1-resistant oligonucleotides of the RNA of influenza A/FW/1/50 virus were separated by two-dimensional gel electrophoresis (Fig. 1*a*) and the large oligonucleotides were numbered 1–44. By coelectrophoresis of the oligonucleotides of the RNAs of FW virus and of other strains (for example, Fig. 1*c*, *g*) it was determined which FW virus-specific spots were missing in a particular pattern (spots missing). Similarly, spots were identified which were absent in the FW virus pattern and present in patterns of the other viruses (additional spots). These spots were labelled 100–162. Oligonucleotides not distinguishable from those present in the FW virus pattern retained the FW virus oligonucleotides designation (1–44). Oligonucleotides present in more than one virus (other than the FW virus) retained the original designation (100–162).

† The oligonucleotide spots of the viruses used in this analysis are identified in Figs 1, 2, 3.

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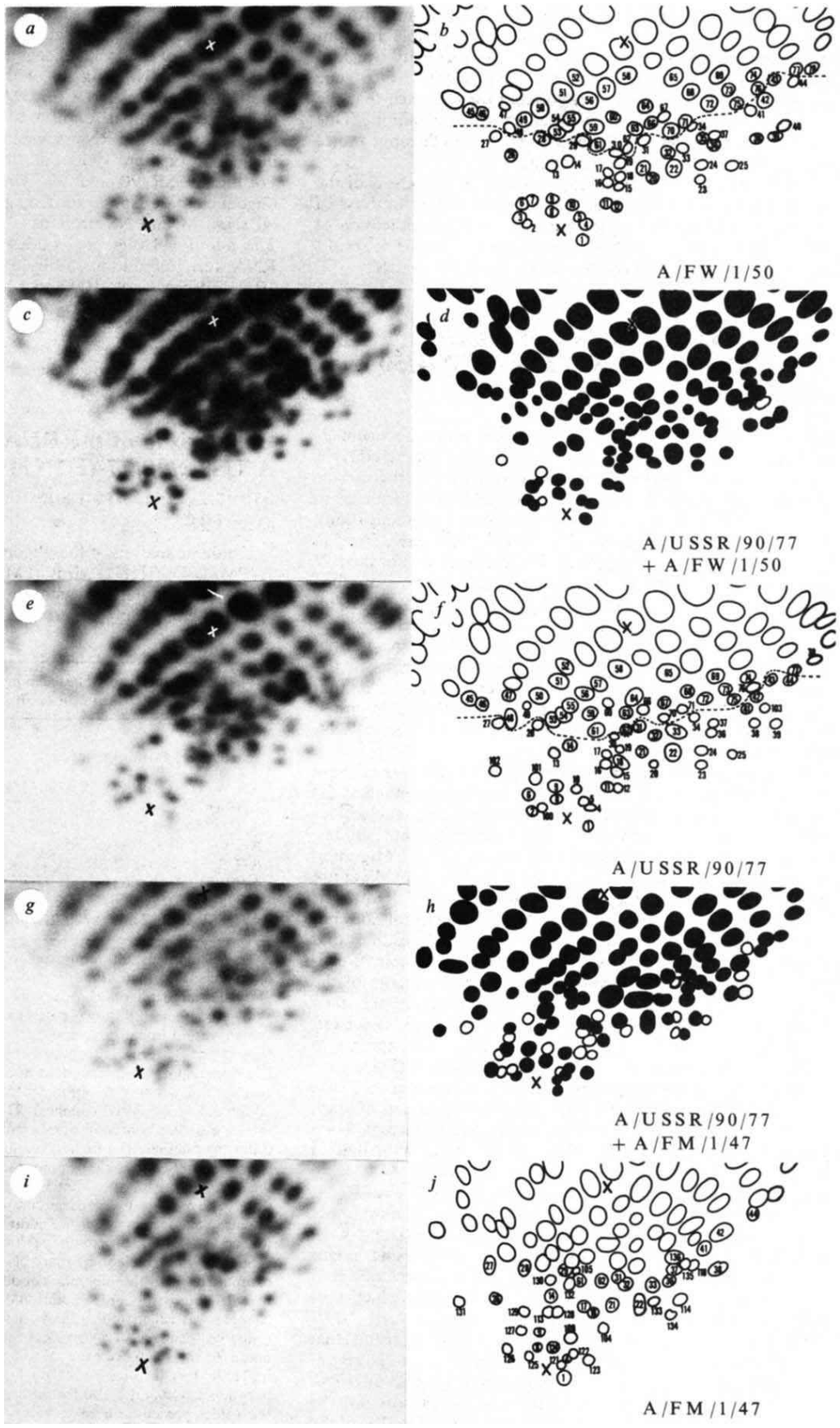

Fig. 1 Oligonucleotide maps of the RNAs of different H1N1 influenza viruses. *a*, RNA of influenza A/FW/1/50 virus. *b*, Diagram of (*a*). *c*, Mixture of the RNAs of influenza A/FW/1/50 and A/USSR/90/77 viruses. *d*, Diagram of (*c*). *e*, RNA of influenza A/USSR/90/77 virus. *f*, Diagram of (*e*). *g*, Mixture of the RNAs of influenza A/USSR/90/77 and A/FM/1/47 viruses. *h*, Diagram of (*g*). *i*, RNA of influenza A/FM/1/47 virus. *j*, Diagram of (*i*). All influenza virus strains were replicated in embryonated eggs. Purified virus was subsequently phenol-extracted[17,18] and the RNA (0.5 μg) was digested with ribonuclease T_1[19]. The resulting oligonucleotides were 5′-end labelled with [γ-^{32}P]ATP using polynucleotide kinase[19] and separated by two-dimensional polyacrylamide gel electrophoresis (refs 20–23 and W. A. Haseltine, personal communication). The first dimension (left to right) was at *p*H 3.5 on a 10% polyacrylamide gel, and the second dimension (bottom to top) was at *p*H 8 in a 21.8% polyacrylamide gel. The large oligonucleotide spots of the RNA of influenza A/FW/1/50 virus are numbered 1–78 (diagram *b*). In patterns of other viral RNAs, those oligonucleotide spots which migrate indistinguishably from spots of FW virus RNA retain the FW virus number. Additional spots (migrating differently from those in the FW virus pattern) are given numbers above 100 (diagram *f*, *j*). For comparison of the RNAs of A/FW/1/50 and A/USSR/90 viruses approximately 80 spots were used because fingerprint maps of the isolated genes were available. In order to compare the oligonucleotide patterns of the total RNAs of all influenza viruses analysed in this study we used only ~40 oligonucleotides in each case (see text and Table 1) (spots below the broken lines in diagrams *b* and *f*). In diagram *d* the black spots (full circles) indicate the oligonucleotides of the A/FW/1/50 virus RNA. The open circles represent the additional spots of the RNA of A/USSR/90 virus. Similarly in diagram *h*, open circles represent the additional A/USSR/90 virus-specific spots and the A/FM/1/47 specific spots are shown as full circles. The positions of the dye markers, xylene cyanole FF and bromophenol blue, are indicated by crosses.

analysis is a more comprehensive method for the comparison of the genetic relatedness of viruses than is possible by conventional measurements of serologic cross-reactivity which in the case of influenza viruses is mediated by only 2 of 8 gene products. In addition, we were looking for evidence that the Russian strains may have emerged by recombination. (One mechanism which has been proposed to explain the appearance of new strains involves recombination[4–8].)

Analysis of the oligonucleotide patterns of the RNAs of the Russian viruses revealed that they are very similar to those of strains isolated in 1950 and that there was no indication of recombination in the origin of the Russian viruses. The results leading to these conclusions are described in this article.

Comparison of RNAs of influenza A/USSR/90/77 (H1N1) and A/FW/1/50 (H1N1) viruses

Serologic examination had revealed that both the haemagglutinin (HA) and the neuraminidase (NA) of the new H1N1 (Russian) isolates were antigenically very similar to the surface glycoproteins of the A/FW/1/50 strain (FW virus) isolated in 1950 (ref. 2 and A. P. Kendal, personal communication). Therefore, we compared the RNA of one Russian (A/USSR/90/77) isolate (USSR/90 virus) with that of the FW virus. Figure 1 shows the two-dimensional patterns of ribonuclease T_1-resistant oligonucleotides of FW and USSR/90 virus RNAs (Fig. 1*a*, *b*, *e*, *f*). Close examination of the maps revealed many similarities between the two RNAs. In addition, analysis of the pattern obtained by coelectrophoresis of the oligonucleotides of both viral RNAs revealed (Fig. 1*c*, *d*) that only four spots (white circles) of the USSR/90 virus migrated differently from corresponding spots of the FW virus RNA (dark circles) (see also Table 1).

In order to estimate the extent of variation between these two viral RNAs the following assumptions have been made. (1) Although large ribonuclease T_1-resistant oligonucleotides make up only a fraction of the total number of nucleotides, they are representative of the entire genome. (2) Migration differences of oligonucleotides in the two-dimensional gel are associated with differences in primary structure. (However, 'common' spots indistinguishable by gel migration do not necessarily possess identical sequences, suggesting that differences detected by this method represent a minimal estimate of the extent of variation.) (3) Differences in the oligonucleotide patterns of closely related RNAs are most likely the result of single base changes (for comparisons see ref. 9). Such a conclusion is probably incorrect when applied to oligonucleotide patterns of more distantly related RNAs.

Based on these assumptions and the data included in Fig. 1 and Table 1 we estimated the minimum number of base changes between the RNAs of FW and USSR/90 viruses. For a comparative analysis of the RNA of FW virus with that of USSR/90 and other viruses, we used only 44 large oligonucleotides (numbered 1–44 in Fig. 1*b*) of FW virus RNA. This number although arbitrary was chosen because these oligonucleotides were most readily distinguishable and were most likely to represent unique sequences. Using oligonucleotide markers[20] we found that the 44 oligonucleotides of FW virus represent a total of 1,077 nucleotides which corresponds to 7.6% of the total genome (molecular weight 4.9×10^6) (ref. 10). Calculations then show (see Table 2 legend) that the minimum base sequence difference between the large oligonucleotides of FW and USSR/90 virus is 8/1,088 or 0.7%.

Our next concern was to determine whether or not the four 'additional' spots (100, 101, 102 and 103 in Fig. 1*d*) of the USSR/90 virus RNA were associated with a single gene. The RNAs of USSR/90 and FW viruses were separated on polyacrylamide gels as previously described[19,20]. The three *P* genes together, the *HA* genes, the *NP* and *NA* genes together, the *M* genes and the *NS* genes were eluted from the gel[20,21] and oligonucleotide maps of the isolated genes were obtained. In the analysis of the isolated segments we compared 78 spots of the FW virus RNA (Fig. 1*b*) with a similar number of spots of the USSR/90 virus RNA (Fig. 1*f*). This number is higher than that used for comparison of total RNAs (44 spots) because the patterns of isolated RNA segments are simpler and permit the distinction of more spots than the maps of total viral RNAs. For the USSR/90 and FW viruses, we assigned individual oligonucleotide spots to the genes coding for P_{1-3}, HA, NP/NA, M and NS proteins, respectively (data not shown). The additional oligonucleotides 100–103 of USSR/90 virus RNA were found to be localised on genes coding for P_{1-3} (spot 101), NP/NA (spots 100 and 103) and M proteins (spot 102). Similar analysis of spots present in the FW virus and not present in the USSR/90 virus confirmed that the detectable oligonucleotide differences between the total RNAs of the two viruses are scattered over the entire genome.

Comparison of the RNA of influenza A/USSR/90/77 (H1N1) virus with RNAs of other H1N1 strains isolated between 1947 and 1956

Subsequent analysis of the oligonucleotide pattern of influenza A/FM/1/47 (H1N1) virus (FM virus, Fig. 1*i*, *j*) isolated in 1947 and comparison with the pattern of USSR/90 virus revealed many more differences than were observed between

Table 2 Minimum no. of base changes amongst large oligonucleotides of the RNAs of different H1N1 influenza viruses

RNAs of strains compared	Mimimum no. of base changes
A/FW/1/50 and A/USSR/90/77	8
A/FM/1/47 and A/USSR/90/77	34
A/C/1/56 and A/USSR/90/77	28
A/FW/1/50 and A/C/1/56	30
A/I/2/50 and A/USSR/90/77	9
A/FW/1/50 and A/I/2/50	5
A/USSR/90/77 and A/HK/123/77	8
A/USSR/92/77 and A/USSR/90/77	6

To calculate the minimum no. of base changes we compared the oligonucleotide maps of different viral RNAs by coelectrophoresis. Only the large oligonucleotides listed in Table 1 were used for this analysis. In comparing two patterns (I and II) there may be 'missing' spots (present in pattern I and not in pattern II) and 'additional' spots (present in pattern II but not in pattern I). Scoring all missing and additional spots as independent mutational events does not take into account that a single mutation could give rise to both a missing and an additional spot (paired changes). Therefore calculations to obtain an estimate of the minimum number of base changes were made in the following manner. The highest number of missing and additional spots which theoretically could be paired was multiplied by a factor of 1.5 on the statistical assumption that 50% of such paired changes are due to a single and 50% to two separate mutational events; to this number the unpaired changes in either the additional or the missing spots category was added (H. D. Robertson, personal communication). For example, using the oligonucleotide map of FW virus RNA as a reference, comparison with the oligonucleotide pattern of USSR/90 virus RNA revealed 6 missing and 4 additional spots. The minimum no. of base changes was calculated to be $4 \times 1.5 = 6$ (paired changes $\times$ 1.5) plus 2 (unpaired changes $\times$ 1.0) giving a total of 8. Similar calculations were made for the RNAs of other H1N1 strains using the data summarised in Table 1. The total no. of nucleotides used in the analysis varied between 1,000 and 1,100 for different strains. It should be noted that the values presented here represent rough estimates of the minimum no. of base changes, because oligonucleotides with different sequences may not necessarily be distinguishable in the conditions used. In particular, the variation between viruses with many oligonucleotide differences (for example between A/FM/1/47 and A/USSR/90/77 virus) is likely to be significantly underestimated.

FW and USSR/90 viruses. More than 20 additional USSR/90 virus specific spots (white circles) can be seen in the mixture of oligonucleotides obtained from FM (black circles) and USSR/90 virus RNAs (Fig. 1*g*, *h*) (see also Table 1). By comparing 44 large oligonucleotides of FM and USSR/90 viruses it can be calculated that the two sets of oligonucleotides differ by a minimum of 34 nucleotides (for calculation see Table 2). In the absence of nucleotide sequence data, errors of underestimation of differences are much more likely with viruses which are more different genetically. First, it is more likely that oligonucleotides which migrate differently may contain more than one base change. Second, a greater proportion of oligonucleotides, which seem to migrate identically, may possess undetected changes than is the case with closely related strains. Therefore, no attempts were made to express the minimum number of nucleotide changes between the FM and USSR/90 virus RNAs in per cent of absolute base sequence differences.

Using the same qualitative approach in comparing oligonucleotide maps we determined the relatedness of the RNA of USSR/90 virus and of a late H1N1 (A/C/1/56) virus (C/1 virus) isolated in 1956 (Fig. 2*a*, *b*). Again, we calculated a minimum of 28 base changes between the large oligonucleotides of these two viral RNAs (Table 2). A similar number of base changes was observed between the oligonucleotides of FW and C/1 viruses isolated in 1950 and 1956, respectively (Table 2).

Finally, as a control we compared the oligonucleotide pattern of (A/I/2/50) virus (I/2 virus), another 1950 isolate, with the patterns of FW and USSR/90 viruses (Fig. 2*c*, *d*). It is evident that the RNA patterns of FW and of I/2 viruses are very closely related (there is a minimum of 5 base changes

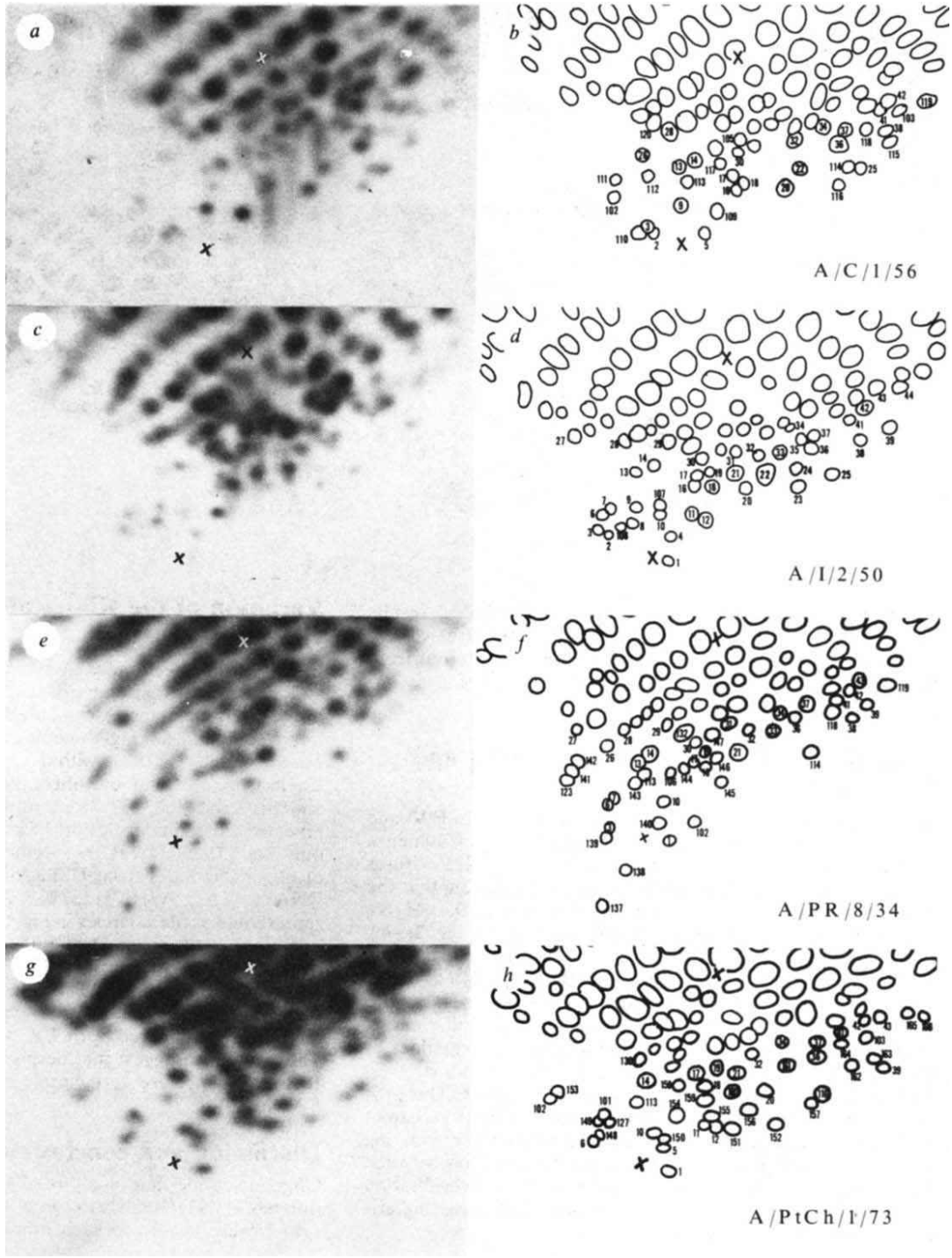

Fig. 2 Oligonucleotide patterns of two H1N1 influenza viruses, of one H0N1 and of one H3N2 influenza virus. *a*, RNA of influenza A/C/1/56 (H1N1) virus. *b*, Diagram of (*a*). *c*, RNA of influenza A/I/2/50 (H1N1) virus. *d*, Diagram of (*c*). *e*, RNA of influenza A/PR/8/34 (H0N1) virus. *f*, Diagram of (*e*). *g*, RNA of influenza A/PtCh/1/73 (H3N2) virus. *h*, Diagram of (*g*). Experimental details as in Fig. 1.

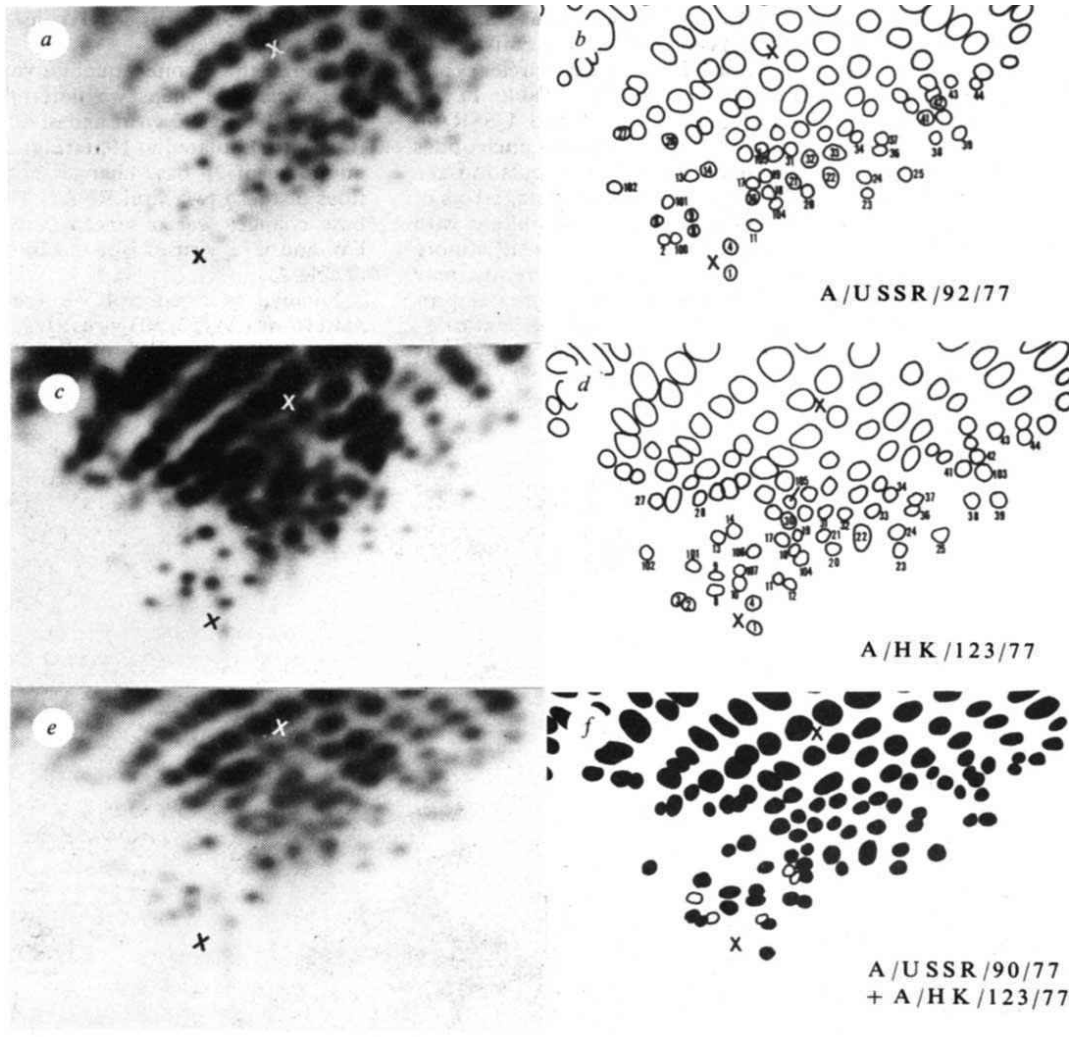

Fig. 3 Comparison of the oligonucleotide patterns of recent H1N1 isolates. *a*, RNA of influenza A/USSR/92/77 virus. *b*, Diagram of (*a*). *c*, RNA influenza A/HK/123/77 virus. *d*, Diagram of (*c*). *e*, Mixture of the RNAs of influenza A/USSR/90/77 (Fig. 1*e*) and A/HK/123/77 viruses. *f*, Diagram of (*e*). Experimental details as in Fig. 1.

between large oligonucleotides) (Table 2). These results confirm our finding that the RNA of USSR/90 virus closely resembles that of strains isolated in 1950 but is different from the RNAs of earlier and later isolates.

Comparison of RNA of H1N1, H0N1 and H3N2 viruses

Comparison of the oligonucleotide maps of the FW and USSR/90 virus RNAs with those of the RNAs of influenza A/PR/8/34 (H0N1) and A/PtCh/1/73 (H3N2) viruses revealed extensive differences in all genes, suggesting that the latter two viruses are not closely related to the 1950 H1N1 strains or to the Russian H1N1 strains (Table 1 and Fig. 2*e–h*). It should be emphasised here that oligonucleotide map analysis is highly valuable for comparing closely related RNA species[9,11–15] and less valuable for determining the absolute relatedness of RNAs with many base sequence differences. Robertson and Jeppesen[9] eluted the large oligonucleotides of the three closely related bacteriophage RNAs compared in their study and subjected them to sequence analysis. This procedure allowed direct determination of the extent of variation. Ultimately the same approach would be needed in comparing influenza virus RNAs to evaluate fully the variation between closely and distantly related strains. In addition, hybridisation data may be useful as a preliminary tool for detecting similarities between distantly related RNAs[16].

Variation of the RNAs of three recent H1N1 viruses

In the previous section we showed that two H1N1 viruses (FW and I/2 viruses) isolated in 1950 possess very similar oligonucleotide maps. A comparably small degree of variation was also found among three Russian (H1N1) viruses which were isolated in 1977 within a period of several months. Coelectrophoresis of the oligonucleotides of USSR/90 virus and of A/HK/123/77 virus, another recent H1N1 isolate, revealed close similarities of the patterns. As shown in Fig. 3*f* only 5 additional USSR/90 specific spots appear in the mixture of oligonucleotides from USSR/90 and A/HK/123/77 virus RNAs. (The A/HK/123/77 virus oligonucleotides are represented as black circles in Fig. 3*f*.) Therefore, the degree of homology between the two isolates is comparable with that between USSR/90 virus and the FW strain which was isolated 28 years ago (Table 2). The oligonucleotide map of a third Russian isolate A/USSR/92/77 virus also showed slight variation when compared with FW virus (Table 2, Fig. 3*a*, *b*). These findings suggest that despite small differences the three Russian isolates are very closely related to each other and to FW virus.

Discussion and conclusion

Oligonucleotide map analysis of the RNAs of three recently isolated H1N1 (Russian) viruses demonstrated that they are very closely related to each other and to two H1N1 viruses

isolated in 1950. In contrast, comparison of the oligonucleotide maps of H1N1 viruses isolated in 1947, 1950 and 1956, revealed extensive genetic differences. These differences in the oligonucleotide maps of the RNAs of viruses isolated within the period of prevalence of one subtype could be due either to the concurrent presence of different variants each of which is genetically conserved, or to multiple mutational events occurring over the entire period. Although the first hypothesis cannot be excluded, for the following reasons we regard the latter explanation to be more likely. First, there is no evidence to show simultaneous prevalence of several markedly different genetic variants belonging to the same subtype. Second, the observation of small differences in the oligonucleotide maps of viruses isolated within a few months of one another suggests that detectable mutations are occurring during epidemic spread over a short period of time.

If we accept the premise that influenza A viruses are subject to repeated mutational events it is extremely difficult to explain why the oligonucleotide maps of strains isolated in 1950 and those of the recent Russian viruses are so strikingly similar. For the reasons stated above it seems unlikely that a 1950 virus survived by normal sequential transmission in the human population without evidence of much more extensive genetic drift. It is also not plausible to speculate that chance backmutations accidentally produced a strain so similar to those isolated in 1950.

It seems much more likely that the genetic information in the Russian viruses has been preserved over the last 25–27 years by some unusual mechanism. Although there is no evidence to support the view, it is possible that influenza viruses are capable of latent or persistent infection in man in conditions in which the genetic information of the virus is highly conserved. Alternatively, the genetic information of the virus could have been preserved by sequential passage in an animal reservoir in which influenza viruses replicate without rapid genetic change. (However, in recent unpublished experiments we observed extensive genetic variation among different influenza viruses isolated over a period of a few years from birds and horses.) Finally, it is possible that a 1950 influenza virus was truly frozen in nature or elsewhere and that such a strain was only recently reintroduced into man.

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Where do general anaesthetics act?

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General anaesthetics were found to have no effect on lipid bilayer structures when studied using X-ray and neutron diffraction. Combined gaseous and aqueous phase solubility data suggested that the primary site of action of general anaesthetics has both polar and nonpolar characteristics, and probably involves protein.

THE induction of general anaesthesia, according to most theories[1–11], involves a structural change in lipid bilayers of nerve cell membranes. Using both X-ray and neutron diffraction, we have looked for such changes in lipid bilayers. Surprisingly, we can detect no change in bilayer structure at clinical concentrations of general anaesthetics. This has led us to reinterpret the evidence previously used to characterise the anaesthetic site. Many attempts have been made to infer the molecular nature of the site from correlations of anaesthetic potency with solubility in various solvents (for a review, see ref. 12). However, data from experiments in which general anaesthetics were administered in the gaseous phase have usually been considered separately from data obtained in aqueous phase experiments. We have combined these data and find an excellent correlation using octanol but very poor correlations using hydrocarbon solvents. From all our results, we conclude that the primary site of action of general anaesthetics has not only apolar but also polar characteristics and probably involves protein.

X-ray and neutron diffraction studies

There is no doubt that sufficiently high concentrations of general anaesthetics will alter membrane structure, and this may account for many of the positive results previously reported with other techniques[13]. Indeed, many general anaesthetics (for example, chloroform and halothane) will dissolve lipid bilayers. It was therefore essential to look for structural changes at clinical concentrations. Also, because we expected only small effects at these low concentrations, it was essential to use the same membrane specimen and diffraction geometry for both control and anaesthetic experiments. Finally, it was necessary to remove the anaesthetic from the sample at the end of the experiment, to check for reversibility. Our solution to these problems was to pass anaesthetics in the gaseous phase over lipid multilayer specimens. In fact, surgical anaesthesia is usually maintained using inhalational anaesthetics, so that the

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