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Antigenic and genetic analyses of eight influenza C strains isolated in various areas of Japan during 1985–9

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SUMMARY

Eight strains of influenza C virus isolated in various areas of Japan between January 1985 and January 1989 were compared using monoclonal antibodies to the haemagglutinin-esterase (HE) glycoproteins and by oligonucleotide mapping of total vRNA. Five of six strains isolated during 1986–9 were closely related to one another and also resembled the virus, C/Aichi/1/81, isolated in 1981 in Aichi prefecture. This suggests that the C/Aichi/1/81-related viruses had an epidemiological advantage over any co-circulating viruses at least during that period. One of two 1985 isolates (C/Nara/1/85) was antigenically indistinguishable from the C/Mississippi/1/80 strain though their oligonucleotide patterns were markedly different from each other. This raises the possibility that C/Nara/1/85 may be a recombinant virus which receives its HE gene from the C/Mississippi/1/80-related parent.

INTRODUCTION

Influenza C virus is widespread in the human population [1, 2] and usually causes a mild upper respiratory illness characterized by fever and long-lasting nasal discharge [3, 4]. The virus contains seven single-stranded RNA segments of negative polarity [5, 6], one of which codes for the surface glycoprotein, haemagglutinin-esterase (HE) [7, 8]. Antigenic analysis with monoclonal antibodies (MAb) against the HE glycoprotein has demonstrated antigenic variation among influenza C strains isolated at different times in different areas [9], but the significance of antigenic variation in the epidemiology of influenza C still remains unclear.

Antigenic drift in influenza A viruses occurs through the accumulation of single point mutations which result in minor alterations of viral surface proteins. Dominant variants emerge from a series of successive mutations and less successful variants disappear [10, 11]. In contrast, analysis of the RNA genomes

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of various influenza C strains suggested that the extent of genetic difference did not correspond to the time of virus isolation and that variants from multiple evolutionary pathways cocirculated at any one time [12–14]. This led to speculation that there might be little if any difference, in the epidemiological potential of influenza C variants. However, we found recently that all three strains (C/Kyoto/41/82, C/Nara/82, C/Hyogo/1/83) isolated in the Kinki district of Japan during the relatively short period from February 1982 to December 1983 were very similar to one another in both genetic and antigenic structure, although they were dissimilar to any of the strains isolated in Japan before 1982 [9, 14]. Furthermore, it was later shown that the three Kinki isolates had a close relationship to the virus isolated in the United States in 1980 (C/Mississippi/1/80) and that they were distinguishable from previous isolates in serological tests with heterogeneous sera [15]. Based on these data, we supposed that influenza C virus closely related to C/Mississippi/1/80, newly introduced into Japan, spread rapidly to the Kinki district during 1982–3 because the virus had epidemiological advantages over the pre-existing viruses.

To extend these observations, we have investigated the antigenic and genetic characteristics of eight human strains of influenza C virus isolated in various areas of Japan during the period from January 1985 to January 1989. The results, presented here, show that five out of six strains isolated later than June 1986 were closely similar to one another in both the reactivity with anti-HE MAb and the oligonucleotide fingerprinting patterns of total vRNA. These data support the idea that there may be considerable differences in the ability to spread in the human population among co-circulating variants of influenza C. We also discuss the possibility that influenza C virus, like influenza A virus, may undergo reassortment of RNA segments in nature.

MATERIALS AND METHODS

Viruses and cells. The following eight strains of influenza C virus, isolated in Japan during 1985–9, were used: C/Nara/1/85, C/Nara/2/85, C/Nara/1/86, C/Yamagata/1/86, C/Nara/1/87, C/Yamagata/6/88, C/Hiroshima/1/88, and C/Hiroshima/1/89. These viruses were recovered from patients with symptoms and signs of acute respiratory illness, two (C/Yamagata/1/86, C/Nara/1/87) from adults and six from young children. They were all isolated from throat swab specimens in the amniotic cavity of 9-day-old embryonated hens' eggs as described previously [3] and then passaged three to five times in the same host. Care was taken to avoid cross-contamination of virus strains, and we showed in preliminary experiments that the different RNA preparations of the same virus strain yielded completely identical oligonucleotide maps. Oligonucleotide maps of the eight influenza C strains, on the other hand, could be distinguished from each other at least in several spots, with the exception of two Hiroshima strains which were isolated only 2 weeks apart in the same city (see below). The four older strains, C/Ann Arbor/1/50 (C/AA/50), C/Mississippi/1/80 (C/MS/80), C/Aichi/1/81, and C/Nara/82, which had been isolated, passaged and propagated in the amniotic cavity of eggs, were also used for comparison. The C/MS/80 strain was

kindly provided by Dr A. P. Kendal (CDC, Atlanta, USA), and Japanese isolates were obtained from the municipal or prefectural institutes of public health. The HMV-II line of human malignant melanoma cells was grown in RPMI 1640 medium containing 10% bovine serum.

MAb and immune sera. A total of 14 anti-HE MAb (11 to C/AA/50 and 3 to C/MS/80) prepared as described elsewhere [15, 16] were used in this study. Competitive binding assays showed that antibodies against the HE of C/AA/50 are directed against six distinct antigenic sites which do not overlap: antibodies J14, Q5, J9, U4, and U9 to site A-1, antibody K16 to site A-2, antibodies U1 and U2 to site A-3, antibody D37 to site A-4, antibody S16 to site B-1, and antibody J6 to site B-2 ([17], unpublished data). The antisera against six different influenza C strains listed in Table 3 were prepared in chickens as described elsewhere [14].

Haemagglutination-inhibition (HI) test. This was done in microtitre plates using 0·5% chicken erythrocytes [3]. The HI titre was expressed as the reciprocal of the highest dilution of antibody which completely inhibited haemagglutination.

Radioimmunoprecipitation (RIP). Twenty-four hours after infection confluent monolayers of HMV-II cells were labelled with [^{35}S]methionine for 2 h and then subjected to immunoprecipitation as previously described [9]. The resulting immunoprecipitates were analysed by SDS–polyacrylamide gel electrophoresis followed by fluorography [18].

Oligonucleotide fingerprinting. Viral genome RNA, extracted from purified virions with SDS–phenol [19], was digested with ribonuclease T1 (Calbiochem–Behring) and 5′ end-labelled with [γ-^{32}P]ATP (New England Nuclear) and polynucleotide kinase (Boehringer–Mannheim). The oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis [14, 20].

Nucleotide sequencing. The HE gene of the C/Nara/1/85 strain was sequenced by the dideoxynucleotide-chain termination method [21] using purified viral RNA as template and synthetic oligonucleotide primers [15]. By using 17 primers described elsewhere [15], the HE gene sequence except for the first 63 nucleotides at the 5′ terminal and 3′ non-coding region (mRNA sense) could be determined.

RESULTS

Antigenic analysis of influenza C strains isolated in Japan during 1985–9. The antigenic structure of eight influenza C virus strains isolated in three prefectures, Nara, Yamagata, and Hiroshima during the period from January 1985 to January 1989 were analysed by HI tests using a panel of 10 anti-HE MAb which showed high titres of HI activity against the homologous strain, C/AA/50 or C/MS/80. As shown in Table 1, one of two 1985 isolates (C/Nara/1/85) from Nara prefecture reacted to high titre with J14, U1, U2, MS2, MS20, and MS22 but failed to react with J9, Q5, U4, and U9, a reactivity pattern virtually identical to the pattern of C/MS/80. Another 1985 isolate (C/Nara/2/85) from the same prefecture showed a reactivity pattern markedly different from that of C/Nara/1/85. The virus also differed from any of the Japanese strains isolated before 1985 in that its reactivity with J14 was extremely low [9]. It was of interest that all six strains isolated between June 1986 and January 1989 were antigenically indistinguishable from one another except that the C/Yamagata/1/86 and C/Yamagata/6/88 strains

Table 1. *Antigenic analysis of influenza C strains isolated in Japan in 1985–9 by HI test with anti-HE MAb*

Viruses	Date of specimen collection	HI titres of the following MAb*									
		Antibodies to C/AA/50									
		Antigenic site A-1					Antigenic site A-3		Antibodies to C/MS/80		
		J14	J9	Q5	U4	U9	U1	U2	MS2	MS20	MS22
Reference strains											
AA/50	—	1024000	256000	32000	32000	128000	256000	128000	<	<	64000
MS/80	—	128000	40	<	40	40	256000	64000	128000	128000	64000
Aichi/1/81	—	512000	160	80	3200	64000	6400	640	<	<	<
1985–9 strains											
Nara/1/85	12 Jan.	128000	<	<	<	<	128000	32000	64000	128000	64000
Nara/2/85	28 June	320	320	3200	40	320	6400	320	<	<	<
Nara/1/86	12 June	1024000	80	80	1600	32000	12800	640	<	<	<
Yamagata/1/86	30 June	512000	80	160	3200	64000	6400	320	<	40	160
Nara/1/87	18 Dec.	512000	80	80	3200	64000	6400	640	<	<	<
Yamagata/6/88	24 Nov.	512000	80	160	1600	32000	6400	640	<	40	80
Hiroshima/1/88	21 Dec.	256000	80	80	3200	64000	6400	320	<	<	<
Hiroshima/1/89	1 Jan.	256000	80	80	3200	64000	6400	320	<	<	<

* <, less than 40.

Table 2. *Antigenic analysis of influenza C strains isolated in Japan in 1985–9 by RIP test with anti-HE MAb*

	Reactivity of the following MAb*									
	Antibodies to C/AA/50								Antibodies to C/MS/80	
	Site A-1			Site A-2	Site A-3	Site A-4	Site B-1	Site B-2		
Viruses	J14	Q5	U4	K16	U1	D37	S16	J6	MS2	MS22
Reference strains										
AA/50	++	++	++	++	++	++	++	++	−	++
MS/80	+	−	++	−	++	++	++	++	++	++
Aichi/1/81	++	−	++	++	++	++	−	++	−	−
1985–9 strains										
Nara/1/85	+	−	++	−	++	++	++	++	++	++
Nara/2/85	−	++	++	++	++	++	++	++	−	++
Nara/1/86	++	−	++	++	++	++	−	++	−	−
Yamagata/1/86	++	+	++	++	++	++	−	++	−	++
Nara/1/87	++	−	++	++	++	++	−	++	−	−
Yamagata/6/88	++	−	++	++	++	++	−	++	−	++
Hiroshima/1/88	++	−	++	++	++	++	−	++	−	−
Hiroshima/1/89	++	−	++	++	++	++	−	++	−	−

* Reactivity was scored as follows: ++, the density of HE band was similar to that of homologous strain; +, the band of HE was less dense than that of homologous strain; −, the band of HE was undetected.

Table 3. *Antigenic analysis of influenza C strains isolated in Japan during 1985–9 by HI test with chicken antiviral sera*

Viruses	HI titres of antisera to					
	AA/1/50	MS/80	Aichi/1/81	Nara/2/85	Nara/1/86	Yamagata/1/86
Reference strains						
AA/1/50	1280	640	640	640	640	640
MS/80	320	10240	640	320	1280	640
Aichi/1/81	1280	2560	10240	2560	10240	10240
1985–9 strains						
Nara/1/85	160	10240	320	320	640	320
Nara/2/85	320	640	1280	2560	1280	640
Nara/1/86	640	1280	5120	2560	10240	5120
Yamagata/1/86	1280	1280	10240	1280	10240	10240
Nara/1/87	1280	2560	10240	2560	10240	10240
Yamagata/6/88	1280	1280	5120	1280	5120	5120
Hiroshima/1/88	640	2560	10240	2560	10240	10240
Hiroshima/1/89	640	1280	5120	1280	10240	10240

were reactive though at low levels with MS20 and MS22. Comparison of the reactivity patterns between these strains and the previously isolated strains revealed that the virus which had been isolated in Aichi prefecture in November 1981 (C/Aichi/1/81) was antigenically very similar to that of the 1986–9 isolates (Table 1).

The antigenic properties of the 1985–9 isolates were further examined in RIP tests using a panel of 10 anti-HE MAb which included four antibodies having little or no HI activity (K16, D37, S16, J6). The results summarized in Table 2 confirmed that the C/Nara/1/85 strain had the same antigenicity as the C/MS/80 strain and that the C/Nara/2/85 strain was antigenically dissimilar to any of the other strains examined. We reported previously that among 15 strains of influenza C virus isolated in Japan and United States between 1947 and 1983, only the C/Aichi/1/81 strain lacked reactivity with MAb S16 [9]. None of the 1986–9 isolates, including C/Yamagata/1/86 and C/Yamagata/6/88, reacted with S16. The reactivity of these strains with the other antibodies was also the same as that of C/Aichi/1/81, except that C/Yamagata/1/86 reacted with Q5 and MS22 and C/Yamagata/6/88 with MS22.

The influenza C virus strains isolated during 1985–9 were further examined by HI tests for reactivity with chicken antisera raised against six different strains (Table 3). It was evident that polyclonal immune sera, like MAb, were able to detect antigenic differences among the strains. Furthermore, the data support the conclusions stated above that C/Nara/1/85 and C/MS/80 were antigenically indistinguishable from each other and that all six strains isolated later than 1986 were antigenically similar to C/Aichi/1/81.

Oligonucleotide fingerprint analysis of influenza C strains isolated in Japan during 1985–9. Genome RNA purified from each of eight Japanese strains isolated during 1985–9 and three previous isolates (C/MS/80, C/Aichi/1/81, C/Nara/82) were subjected to T1-oligonucleotide fingerprinting, and the resulting maps were compared. It was found that five of six 1986–9 isolates (C/Nara/1/86,

C/Nara/1/87, C/Yamagata/6/88, C/Hiroshima/1/88, C/Hiroshima/1/89) exhibited oligonucleotide patterns remarkably similar to one another (Fig. 1, oligonucleotide map of C/Hiroshima/1/88 not shown). When pairwise comparisons were made using about 60 large oligonucleotides that migrated below the broken lines marked on Fig. 1, they differed from each other by fewer than eight oligonucleotide spots (Table 4). The C/Yamagata/1/86 strain, though different in larger numbers of spots, still showed an overall pattern of large oligonucleotides fairly similar to the patterns of the above five strains (Fig. 1). Moreover, it was observed that the C/Aichi/1/81 strain yielded an oligonucleotide map with considerable similarity to the maps of six 1986–9 isolates (Table 4 and Fig. 1), as expected from the pronounced similarity in their antigenicity.

Fig. 2 shows the oligonucleotide fingerprints of C/Nara/82, C/Nara/1/85, and C/Nara/2/85. The C/Nara/82 strain has been shown previously to possess the same antigenicity as the C/MS/80 strain [9, 15], and the latter strain was observed here to be indistinguishable antigenically from the C/Nara/1/85 strain (Tables 1–3). It was unexpected, therefore, that the oligonucleotide pattern of C/Nara/1/85 was largely different from those of C/MS/80 and C/Nara/82; the patterns of the latter two isolates were very similar to each other, with a difference of only six oligonucleotide spots (Table 4). Similarity was evident in the maps of C/Nara/1/85 and C/Nara/2/85 despite the remarkable difference in the antigenicity of their HE glycoproteins (Tables 1–3).

Sequence analysis of the HE gene of C/Nara/1/85. The data presented above showed that C/Nara/1/85 and C/MS/80 or C/Nara/82, while exhibiting the same reactivity with anti-HE MAb and antiviral sera (Tables 1–3), could be distinguished in fingerprinting patterns by a number of large oligonucleotides (Table 4). To rule out the possibility that despite the identical antigenicity, there might be only a low degree of nucleotide sequence homology between the HE genes of C/Nara/1/85 and C/MS/80 or C/Nara/82, the sequence of the C/Nara/1/85 HE gene was determined and then compared with the previously determined sequences of the C/MS/80 and C/Nara/82 HE genes [12, 15]. The results (Table 5) showed that the sequence of C/Nara/1/85 was highly homologous to those of C/MS/80 and C/Nara/82, with differences of 1·3 and 1·1%, respectively; these values were comparable to or even lower than the nucleotide difference (1·6%) observed between the latter two strains. The C/MS/80-related viruses (including C/Nara/82) have been shown to be unique in that they had the HE genes lacking three nucleotides at position 641–643, leading to the loss of one amino acid in the HE1 region of the molecule [12, 15]. The deletion of three nucleotides in these positions was detected in the gene of C/Nara/1/85. Additionally, comparison of the deduced amino acid sequences showed that the *HE protein of C/Nara/1/85 differed from the HEs of C/MS/80 and C/Nara/82 in* only 8 and 2 amino acids, respectively (Table 5). Consequently, the remarkable differences observed in the oligonucleotide maps of C/Nara/1/85 and C/MS/80 or C/Nara/82 are difficult to explain by nucleotide sequence differences in their HE genes.

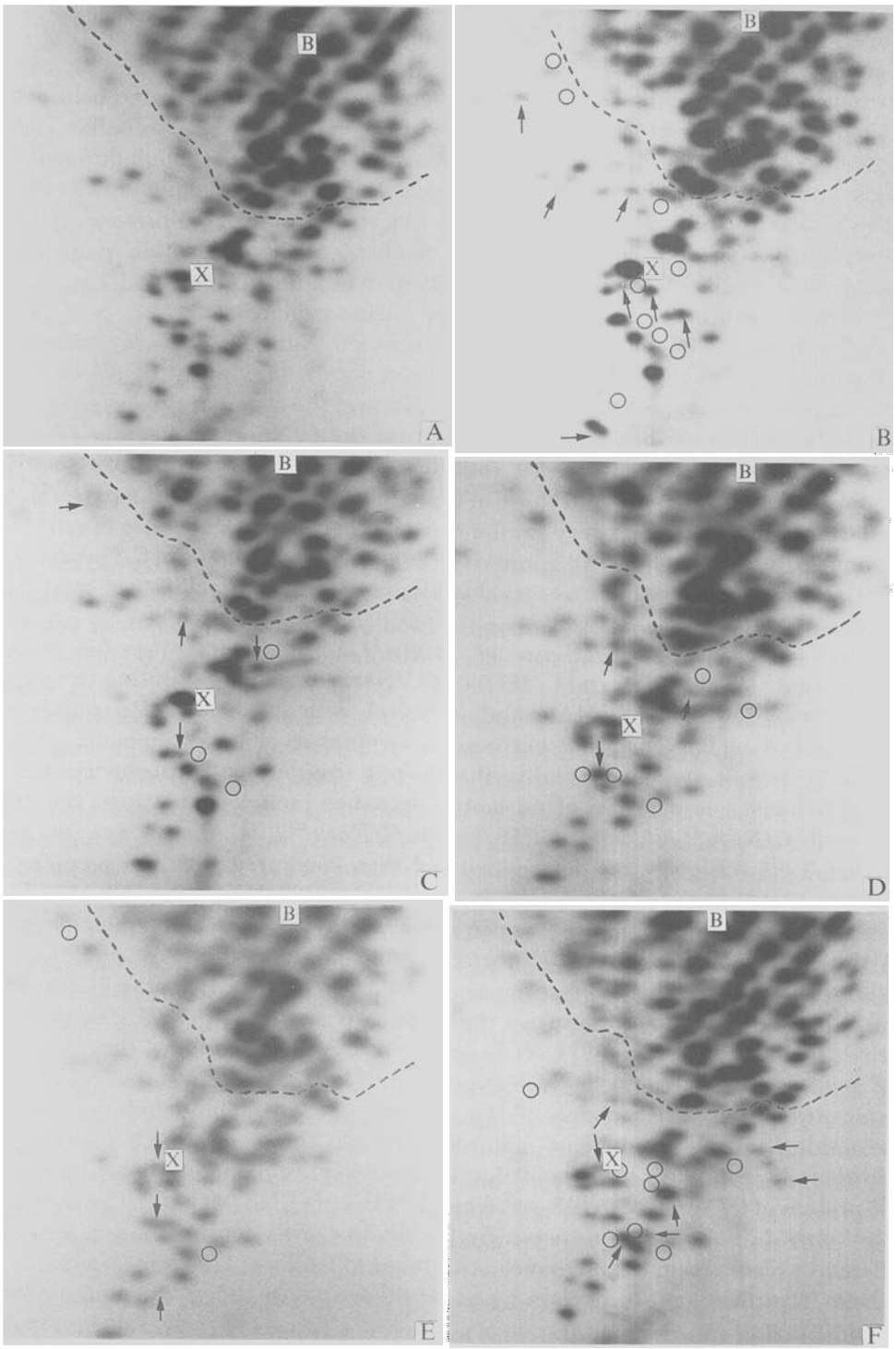

Fig. 1. Oligonucleotide fingerprints of C/Nara/1/86 (A), C/Yamagata/1/86 (B), C/Nara/1/87 (C), C/Yamagata/6/88 (D), C/Hiroshima/1/89 (E), and C/Aichi/1/81 (F). The oligonucleotide spots absent in C/Nara/1/86 but present in each of the other strains are indicated by arrows, and the spots present in the former but absent in the latter are indicated by open circles. The positions of the dye markers, xylene cyanol and bromphenol blue, are indicated by X and B, respectively.

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Table 4. *Pairwise comparison of oligonucleotide fingerprints between different influenza C isolates**

	MS/80	Aichi/1/81	Nara/82	Nara/1/85	Nara/2/85	Nara/1/86	Yamagata/1/86	Nara/1/87	Yamagata/6/88	Hiroshima/1/88	Hiroshima/1/89
MS/80	—										
Aichi/1/81	39	—									
Nara/82	6	35	—								
Nara/1/85	34	36	36	—							
Nara/2/85	35	39	32	15	—						
Nara/1/86	36	15	31	33	38	—					
Yamagata/1/86	41	15	40	35	34	16	—				
Nara/1/87	37	14	33	32	37	7	12	—			
Yamagata/6/88	37	13	32	31	36	8	18	5	—		
Hiroshima/1/88	38	17	35	34	35	5	16	6	5	—	
Hiroshima/1/89	38	17	35	34	35	5	16	6	5	0	—

* Each number indicates the sum of missing and additional oligonucleotide spots observed in the maps of two given strains.

DISCUSSION

This study was undertaken to examine the possibility that an influenza C variant closely related to C/MS/80, introduced into Japan shortly before 1982 [15], may have spread rapidly over the country. However, the data presented here showed that of eight influenza C strains isolated in Japan during 1985–9, only the C/Nara/1/85 strain was antigenically indistinguishable from C/MS/80. A high degree of nucleotide sequence homology was observed between the HE genes of C/Nara/1/85 and C/MS/80, but total vRNA from these isolates yielded oligonucleotide maps greatly different from each other. Thus no evidence was obtained that the virus closely related to C/MS/80 became prevalent throughout Japan as one of the dominant influenza C variants. Rather, the data showed that of the six strains isolated between June 1986 and January 1989, five (including C/Yamagata/6/88) were similar to one another both in the patterns of reactivity with anti-HE MAb and chicken anti-sera and in the oligonucleotide maps, and that these five strains were closely related antigenically and genetically to the C/Aichi/1/81 strain. The remaining strain (C/Yamagata/1/86) was also related, though less closely, to C/Aichi/1/81. Moreover, four of five influenza C strains, isolated in Sendai City during the winter of 1990–1, were indistinguishable from C/Aichi/1/81 in reactivity with a panel of anti-HE MAb (unpublished data). These observations suggest strongly that influenza C viruses related to C/Aichi/1/81 have been dominant in Japan since 1986. The 1986–9 strains, all related to C/Aichi/1/81, could be distinguished with heterogeneous sera in HI tests from two 1985 strains (C/Nara/1/85, C/Nara/2/85) as well as from two older strains (C/AA/50, C/MS/80) (Table 3), a finding which raises the possibility that the C/Aichi/1/81-related viruses may have possessed a selective advantage in the

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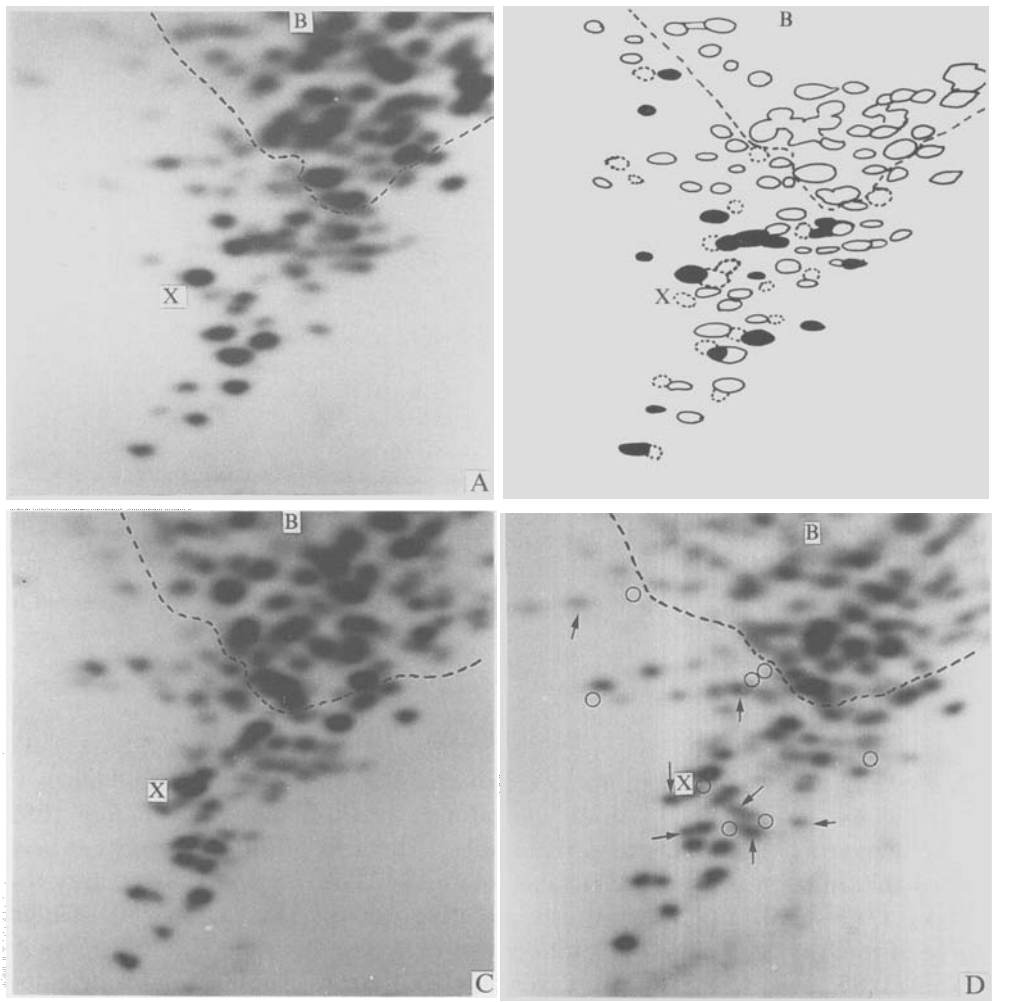

Fig. 2. Oligonucleotide fingerprints of C/Nara/82, C/Nara/1/85 and C/Nara/2/85. (A) map of C/Nara/82; (B) diagram of (A); (C) map of C/Nara/1/85; (D) map of C/Nara/2/85. In (B) the oligonucleotides detected in C/Nara/82 but not in C/Nara/1/85 are indicated by filled-in circles, and the oligonucleotides found in C/Nara/1/85 but missed in C/Nara/82 are circled by dotted lines. In (D) the oligonucleotide spots absent in C/Nara/1/85 but present in C/Nara/2/85 are indicated by arrows, and the spots present in the former but absent in the latter are indicated by open circles.

presence of immunity to the previously prevalent influenza C strains. To validate this, however, antigenic properties of the C/Aichi/1/81-related viruses must be compared with those of a larger number of viruses prevalent before 1986, by using a larger number of heterogeneous sera including human sera collected around 1986. It is also possible that some factors other than immune pressure such as enhanced replication in the upper respiratory epithelium and increased transmissibility might be responsible for high prevalence of the C/Aichi/1/81-related viruses.

Antigenic analysis with polyclonal antibodies revealed that all influenza C isolates examined so far were highly cross-reactive [14, 22, 23] suggesting that

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Table 5. *Differences in nucleotide and deduced amino-acid sequences of HE genes of C/MS/80, C/Nara/82, and C/Nara/1/85**

Nucleotide differences				Amino acid differences			
Base no.†	MS/80	Nara/82	Nara/1/85	Amino acid no.†	MS/80	Nara/82	Nara/1/85
108	C	T	C				
126	C	T	T				
400	T	T	A	127	Tyr	Tyr	Asn
411	C	C	T				
561	G	A	G				
625	G	G	A	202	Glu	Glu	Lys
710	A	G	G	230	Lys	Arg	Arg
750	C	T	T				
769	A	C	C				
774	A	A	G				
862	A	T	T	281	Thr	Ser	Ser
895	C	A	A	292	Leu	Ile	Ile
901	C	T	T				
903	G	A	A				
936	A	G	G				
939	A	G	A				
945	T	C	T				
954	T	A	A				
960	G	A	G				
992	T	C	C	324	Leu	Pro	Pro
1260	T	C	T				
1305	T	C	T				
1320	C	C	A				
1464	T	C	T				
1476	A	G	A				
1488	A	A	G				
1491	G	A	A				
1497	A	G	G				
1554	A	G	A				
1570	A	T	T	517	Thr	Ser	Ser
1590	C	T	T				
1596	T	T	C				
1623	A	G	A				
1671	G	A	G				
1710	G	A	A				
1824	G	G	A				
1869	A	T	A				
1930	A	G	G	637	Thr	Ala	Ala

* Only positions that differed among C/MS/80, C/Nara/82, and C/Nara/1/85 are shown. The sequences for C/MS/80 and C/Nara/82 have been published previously [12, 15].

† Numbered according to the positive strand sequence of the C/AA/50 strain [12].

‡ Numbered according to the amino acid sequence of the C/AA/50 strain [12].

influenza C virus is antigenically much more stable than influenza A and B viruses. It was observed here that five strains isolated over a period of about 7 years (C/Aichi/1/81, C/Nara/1/86, C/Nara/1/87, C/Hiroshima/1/88, C/Hiroshima/1/89) displayed identical patterns of reactivity with a panel of 14 anti-HE MAb. Furthermore, as stated above, several influenza C strains with antigenicity

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indistinguishable from that of C/Aichi/1/81 were isolated in Sendai city during December 1990–January 1991 (unpublished data). It appears, therefore, that influenza C virus can survive in a country for 9 years or longer without changing the specificity of its HE antigen.

The HE gene of C/Nara/1/85 was shown to have a high nucleotide sequence homology (98·9%) with that of C/Nara/82, a virus isolated 3 years before in the same prefecture. Oligonucleotide fingerprinting analysis performed on the total vRNA revealed, however, that maps of the two isolates were markedly different from each other. These curious observations could be accounted for if the C/Nara/1/85 strain arose by a process of genetic reassortment. It has been demonstrated previously that influenza C viruses, like influenza A and B viruses, undergo reassortment of RNA segments *in vitro* [5]. Guo and Desselberger [6] showed that the genomes of two swine influenza C strains isolated on the same day at the same place differed by a number of mutations which were all located in RNA segments 1 and 2, and suggested that they were genetically related by a reassortment event. In order to examine the hypothesis that the C/Nara/1/85 strain may be a recombinant virus which received its HE gene from the virus closely related to C/Nara/82, sequence analysis of other genes from C/Nara/1/85 and C/Nara/82 are now in progress.

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