Archives of Virology 86, 197—211 (1985)

Archives of Virology

Characterization of a 1980-Swine Recombinant Influenza Virus Possessing H1 Hemagglutinin and N2 Neuraminidase Similar to that of the Earliest Hong Kong (H3N2) Virus

By

K. Nerome, Y. Yoshioka[1], S. Sakamoto[2], H. Yasuhara[3], and A. Oya[1]

[1] Department of Virology and Rickettsiology, National Institute of Health, Shinagawa-ku, Tokyo

[2] The Chemo-Sero-Therapeutic Research Institute, Shimizu, Kumamoto

[3] Kyoto Biken Laboratories, Uji, Kyoto, Japan

With 3 Figures

Accepted March 11, 1985

Summary

A recombinant (H1N2, formerly Hsw1N2), A/swine/Ehime/1/80 was found to possess antigenic, biological and genomic characteristics different from those of a previous A/swine/Kanagawa/2/78 (H1N2) strain (22). Five monoclonal antibodies to A/NJ/8/76 definitely differentiated the hemagglutinin molecules of the former virus from the latter, showing that these viruses differed, at least, at two antigenic determinants. Neuraminidase-inhibition tests with monoclonal antibodies to different H2N2 and H3N2 viruses revealed that the A/swine/Ehime/1/80 strain contained a neuraminidase very similar to that of the late human Asian (H2N2) and the earliest Hong Kong (H3N2) viruses. Growth comparison of swine and human isolates indicated that A/swine/Ehime/1/80 and A/swine/Shizuoka/1/78 (H1N1) failed to grow at 42° C, while A/swine/Kanagawa/2/78 and its possible parental virus, A/swine/Kanagawa/4/78 (H1N1) replicated efficiently at this stringent temperature. These results revealed that the viruses having growth characteristics similar to those of avian influenza virus were present in the swine population. RNA analysis by oligonucleotide mapping suggested that A/swine/Ehime/1/80 may be a recombinant between A/swine/Shizuoka/1/78-like and A/Aichi/2/68 (H3N2)-like viruses. To further determine the gene constellation of this recombinant virus, DNA-RNA hybridization was performed by using DNA segments complementary for swine

(H1N1) virus RNA and the entire RNAs of three viruses. The molecular hybridization could define the genomic composition of the recombinant, indicating that only the neuraminidase gene of this virus is derived from the earliest Hong Kong (H3N2)-like virus and remaining seven genes from swine (H1N1) virus.

Introduction

While outbreaks of clinical influenza had been recorded in many countries before 1900 (37), an epidemiological relationship between human and animal influenza was first suggested based on 1918/19 outbreaks of human and swine influenza in the Mid-West of the U.S.A. by Koen (14). The subsequent virus isolation from swine in 1930 (31, 32) and confirmatory serological evidence of infection of man with this virus established with certainty a close relationship between human and swine influenza (8, 33), indicating that swine (H1N1) virus was the causative agent which had been prevalent in man during the period of 1918—1924. Since then swine have been considered largely as a key to understand the human influenza epidemics, but the sudden appearance of A/Hong Kong (H3N2) influenza virus in man brought research worker's attention to the bird world due to the following evidence: The hemagglutinin gene of this pandemic strain appeared to be derived from an avian virus but the remaining seven genes were from the preceeding human Asian (H2N2) virus (30). Recently a large-scale virological surveillance of lower animals and birds revealed that numerous influenza A virus subtypes and their variants, which were antigenically related to human epidemic strains, have been conserved in the avian population (11, 13, 17), suggesting potential hosts for elucidation of natural history of influenza virus. However, antigenic and genomic analyses of Asian and A/Hong Kong viruses strongly suggested that genetic reassortment may be an essential mechanism by which new pandemic viruses appear in man (7, 16, 30). With the exception of one report (4), there is no direct evidence that men have been infected naturally with avian influenza viruses without genetic reassortment. Although the viruses distributed in the avian population may act as the source of new virus which produces pandemic influenza in man, it may be that a change of gene composition of these viruses is necessary to cross the host barrier and infect the human population. It is also of interest that swine have been infected with the viruses originating from avian population (26). Moreover, numerous isolations of H3N2 virus from swine, which were related antigenically and genetically to different variants of human virus (10, 15, 21, 29, 34, 35, 36) have made swine an important reservoir as the place where genetic reassortment could occur in nature. In a previous paper we revealed that a recombinant isolated from a pig contained the neuraminidase gene from a human Victoria (H3N2)-like strain and the remainder from swine (H1N1) virus (22). In 1980 some (H.Y.)

of us also isolated another recombinant virus possessing H1 (sw1) hemagglutinin and N2 neuraminidase from a pig in Ehime prefecture, Japan (38).

In the present communication we describe antigenic, biological and genomic characteristics of the above recombinant virus from 1980 in Japan and discuss the significance of isolation of this virus on the basis of comparison with characteristics of a previous recombinant from 1978 (22).

Materials and Methods

Viruses

Ten H1N1 (formerly Hsw1N1) and two H1 (sw1)N2 recombinant viruses isolated from swine in Japan during the period of 1978—1980 were employed in the present studies: A/sw/Shimane/1/78 (H1N1), A/sw/Shizuoka/1/78 (H1N1), A/sw/Kanagawa/4/78 (H1N1), A/sw/Niigata/1/78 (H1N1), A/sw/Fukuoka/1/78 (H1N1), A/sw/Toyama/1/78 (H1N1), A/sw/Sawara/1/78 (H1N1), A/sw/Kobe/1/80 (H1N1), A/sw/Kobe/3/80 (H1N1), A/sw/Kobe/4/80 (H1N1), A/sw/Kanagawa/2/78 (H1N2), A/sw/Ehime/1/80 (H1N2). In addition to the above viruses, the following human and swine viruses were subjected to different analyses: A/Aichi/2/68 (H3N2), A/Kumamoto/22/76 (H3N2), A/sw/Wadayama/5/69 (H3N2), A/sw/Hong Kong/3/76 (H3N2), A/sw/Hong Kong/4/76 (H3N2), A/sw/Hong Kong/6/76 (H3N2), A/sw/Bangkok/9/79 (H3N2). All viruses were grown in the allantoic cavity of 11-day-old fertile hen's eggs.

Hemagglutination-Inhibition (HI) Tests

HI tests were performed in a reduced volume using disposable microtiter-U-plates, and 0.5 per cent chicken red blood cells.

Neuraminidase-Inhibition (NI) Tests

NI tests with monoclonal antibodies were done according to the methods described by AYMARD-HENRY *et al.* (2), but the diluent contained 0.5 per cent Triton X-100 to expose efficiently the antigenic determinants of neuraminidase molecules (28).

Monoclonal Antibodies

All monoclonal antibodies to hemagglutinin of A/NJ/8/76 (H1N1), and to neuraminidases of different H2N2 and H3N2 viruses were kindly provided by Dr. R. G. Webster.

Growth Experiments

MDCK cell monolayers were used in comparative growth tests at 37° and 42° C, and experimental conditions were the same as in a previous report (23).

Oligonucleotide Mapping

The whole viral RNAs were extracted from highly purified egg-grown viruses by the hot-phenol method (24, 27). Approximately 5 μg amounts of these RNAs were digested with T1 ribonuclease (Sankyo Co., Ltd., Japan) and the 5′-ends were labelled with (γ-^{32}P) ATP (>5000 Ci/mmol; Amersham, England) using T4 polynucleotide kinase (Boehringer, Mannheim) (3, 19). Two dimensional separation of the labelled oligonucleotides were performed by the methods described previously (6, 19, 25). However, Tris-borate buffer (50 mM) was used in the second dimension of electrophoresis, and the second dimension was conducted by running from top to bottom (23).

DNA-RNA Hybridization

For molecular hybridization, DNA complementary (c) to the total RNAs of A/sw/Kanagawa/4/78 (H1N1) was synthesized in the presence of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, Fla., U.S.A.) by using a dodecadeoxyribonucleotide primer (AGCAAAAGCAGG) and (α-^{32}P) dCTP (Amersham, England) as described (5).

Synthesized cDNA was fractionated on 7 M-urea-4 per cent polyacrylamide gel electrophoresis and the separated cDNA segments were eluted according to the methods described in a previous paper (22). Six ng of each cDNA segment was used in liquid DNA-RNA hybridization tests, and experimental conditions were described in previous papers (1, 22). Increased concentrations of whole viral RNAs were used in these tests to obtain the desired Crt (concentration of RNA in mol/l $\times$ time in seconds).

Results

Antigenic Analysis of the Hemagglutinin of the Isolates from Swine

Although a recombinant, A/sw/Ehime/1/80 strain was already identified as H1 (sw1) N2 subtype, to know the possible parental virus we further compared in detail the hemagglutinin antigen of this recombinant and 9 strains of swine (H1N1) influenza virus using monoclonal antibodies and post-infection ferret serum. The results are shown in Table 1. Hemagglutinating activity of all viruses was inhibited to similar levels by a ferret antiserum, indicating close antigenic relatedness to each other. However, five mono-

Table 1. *Antigenic analysis of the hemagglutinin antigens of a recombinant and swine influenza viruses in Japan by post-infection ferret serum and monoclonal antibodies*

Test viruses	Ferret serum	HI titers					Antigenic subtypes
		Monoclonal antibodies					
	A/NJ/ 8/76	6/1a	117/2	72/3	40/3	36/3	
sw/Ehime/1/80	1280	6400	—[a]	—	3200	3200	H1N2
sw/Shimane/1/78	1280	6400	—	—	3200	3200	H1N1
sw/Shizuoka/1/78	1280	6400	—	—	1600	3200	H1N1
sw/Niigata/1/78	1280	6400	6400	6400	3200	3200	H1N1
sw/Fukuoka/1/78	1280	6400	6400	6400	3200	3200	H1N1
sw/Toyama/1/78	1280	6400	6400	6400	3200	3200	H1N1
sw/Sawara/80	1280	6400	6400	6400	1600	3200	H1N1
sw/Kobe/1/80[b]	2560	6400	6400	6400	1600	3200	H1N1
sw/Kobe/3/80[b]	1280	6400	6400	6400	1600	1600	H1N1
sw/Kobe/4/80[b]	1280	6400	6400	6400	1600	1600	H1N1

Values given are expressed as the reciprocal of the terminal serum or antibody dilution inhibiting hemagglutination of the virus antigens tested.

[a] Less than 100

[b] Strains were isolated from pigs imported from the U.S.A.

clonal antibodies to hemagglutinin of A/NJ/8/76 revealed their slight antigenic differences, showing that two antigenic variants of swine (H1N1) virus have been co-circulating in the swine population of Japan. For reference, Table 1 also shows data on A/sw/Shizuoka/1/78, which was described in a previous paper (22). Of 9 swine H1N1 viruses 7 reacted with all monoclonal antibodies, but two isolates, A/sw/Shizuoka/1/78 and A/sw/Shimane/1/78 failed to react with two monoclones (117/2, 72/3). A/sw/Ehime/1/80 virus

Table 2. *Comparison of the antigenic structure of the neuraminidase subunits of a recombinant and H3N2 influenza viruses isolated from swine and man on the basis of inhibition patterns with monoclonal antibodies*

			Neuraminidase inhibition patterns to the following viruses:									
			Swine isolates						Human isolates			
Antibody to	Clones	Homologous NI titers	Ehime/1/80	Wadayama/5/69	HK/3/76	HK/6/76	HK/4/76	Bangkok/9/79	Tokyo/1/67	Aichi/2/68 [b]	Tokyo/6/73 [b]	Kumamoto/22/76 [b]
Jap/305/57	78/4	1100	— [a]	—	—	—	—	—	—	—	—	—
	102/2	700	—	—	—	—	—	—	—	—	—	—
	136/5	1100	—	—	—	—	—	—	—	—	—	—
	117/2	400	—	—	—	—	—	—	—	—	—	—
Tokyo/3/67	S 10/1	700	1024	1024	1024	1024	—	—	+	+	—	—
	16/8	500	1024	1024	256	512	—	—	+	+	—	—
	23/9	1000	1024	1024	512	512	—	—	+	+	—	—
	25/4	2900	4096	1024	1024	1024	—	—	+	+	—	—
	S 25/4	100	1024	512	1024	512	—	—	+	+	—	—
Port Chalmers/ 1/73	27/5	160	32	16	—	32	64	64	—	+	+	+
Vict/3/75	12/2	300	—	—	—	—	512	512	—	—	+	+
	27/3	250	—	—	—	—	512	256	—	—	+	+
	21/3	250	—	—	—	—	512	1024	—	—	+	+
Texas/1/77	18/3	600	—	8	4	4	128	128	+	+	+	+
	19/1	800	—	—	—	—	8	4	+	+	+	+
	67/3	100	8	—	—	—	8	4	+	+	+	+
	69/1	1000	—	—	—	—	—	4	—	—	+	—
	78/1	200	—	—	—	—	4	4	—	—	+	+
	88/2	200	—	—	—	—	4	—	—	—	+	+
	123/3	500	—	—	—	—	—	256	—	—	+	+

[a] Less than 4. NI titers expressed as the reciprocal of the terminal antibody dilution inhibiting 50 per cent neuraminidase activity of viruses

[b] For comparison, Table also shows neuraminidase inhibition patterns of human H3N2 viruses presented by us (22)

was also found to contain hemagglutinin antigen identical to the latter two swine isolates. These results strongly suggested that the hemagglutinin antigen of a recombinant virus from 1980 was derived from a A/sw/Shizuoka/1/78- or A/sw/Shimane/1/78-like virus variant.

Comparative Antigenic Analysis of the Neuraminidase of a Recombinant and H3N2 Isolates from Swine and Man

In order to search carefully for clues in respect to the possible origin of the recombinant from 1980, a panel of monoclonal antibodies to human H2N2 and H3N2 viruses were used in the following NI tests. Though monoclones to early Asian virus did not react with all viruses examined, 5 monoclones against a later one, A/Tokyo/3/67, clearly detected antigenic characteristics of neuraminidase of early human Hong Kong (H3N2) viruses (Table 2). Conversely, neuraminidase-inhibition patterns with monoclonal antibodies to two Hong Kong strains, A/Victoria/3/75 and A/Texas/1/77 differentiated late Asian or early Hong Kong viruses from 1973-H3N2 and later viruses. All monoclonal antibodies to A/Tokyo/3/67 inhibited neuraminidase activity of A/sw/Ehime/1/80 and three H3N2 isolates from swine between 1969 and 1976, as did late Asian and early human H3N2 viruses. For reference, Table 2 also includes neuraminidase-inhibition profiles of human H2N2 and H3N2 viruses. A/sw/Ehime/1/80 virus was found to possess a neuraminidase antigenically closely related to late Asia and early Hong Kong viruses. They differed only at a few sites recognized by monoclonal antibodies to A/Texas/1/77. It was also of interest to know that H3N2 viruses similar to human early Hong Kong virus such as A/Aichi/2/68 have been circulating in swine populations, showing that neur-

Table 3. *Replication of swine and human influenza viruses at 37° and 42° C*

	HA and infectivity titers					
	Days					
	37° C			42° C		
Virus strains	1	2	3	1	2	3
sw/Shizuoka/1/78 (H1N1)	2	16	32	< (−)[b]	< (−)	< (−)
sw/Ehime/1/80 (H1N2)	<[a]	8	16	< (−)	< (−)	< (−)
sw/Kanagawa/4/78 (H1N1)	16	32	64	2 (3.46)	32 (3.55)	16 (4.10)
sw/Kanagawa/2/78 (H1N2)	64	32	64	16 (5.18)	32 (4.86)	64 (4.43)
Aichi/2/68 (H3N2)	32	32	64	< (−)	< (−)	< (−)
Kumamoto/22/76 (H3N2)	16	64	256	< (−)	< (−)	< (−)

Growth experiment and plaque titration were done in MDCK cells in the presence of acetyltrypsin (5 mcg/ml, Sigma).

[a] HA titer less than 2

[b] Log_{10} PFU/ml less than 1.5. Values given in parenthesis represent log_{10} PFU/ml

aminidase of A/sw/Ehime/1/80 was antigenically also very similar to two H3N2 swine isolates from 1976.

Growth Comparison of the Isolates at High Temperature

To compare the growing ability of two recombinants and other swine and human isolates, confluent MDCK cell monolayers were infected with approximately 0.01 MOI of different viruses and were kept at 37° and 42° C. Table 3 shows the growth characteristics of the different strains obtained in multiple cycle conditions. It can be seen that all viruses replicated at 37° C and released hemagglutinin into the culture medium. Under this temperature-condition no notable differences in the final yield based on hemagglutinating activity, were observed. In contrast to 37° C, two recombinant viruses showed different behavior in their growth at 42° C. Of the viruses tested, a 1978-recombinant, A/sw/Kanagawa/2/78 and its possible parental virus, A/sw/Kanagawa/4/78 (H1N1) grew well at 42° C and produced hemagglutinin which corresponded to infectious progeny virus. This is the first evidence indicating the presence of virus capable of growth of high temperatures in the swine population. However, the remainder failed to replicate at 42° C. These results led us to compare the efficiency of plaque formation of swine and human isolates at both temperatures. As shown in Table 4, two swine isolates, A/sw/Kanagawa/2/78 (H1N2) and A/sw/Kanagawa/4/78 (H1N1) formed plaques with a high efficiency at 42° C, whereas the remaining 4 viruses including a 1980-recombinant did not produce plaques under this condition. These results led to a possibility that the parental swine (H1N1) virus of A/sw/Ehime/1/80 is different from that of the 1978-recombinant.

RNA Analysis by Oligonucleotide Mapping

The differences of antigenic and biological properties of the two recombinant viruses suggested that these viruses were derived from different

Table 4. *Efficiency of plaque formation of swine H1N1 and a recombinant, and human H3N2 viruses at 37° and 42° C*

Virus strains	Plaque titer ($\log_{10}$ PFU/ml)	
	37° C	42° C
sw/Kanagawa/2/78 (H1N2)	5.88	4.35
sw/Kanagawa/4/78 (H1N1)	7.67	4.90
sw/Shizuoka/1/78 (H1N1)	5.60	<[a]
sw/Ehime/1/80 (H1N2)	5.27	<
Aichi/2/68 (H3N2)	7.57	<
Kumamoto/22/76	7.52	<

Parallel plaque titrations at 37° and 42° C were performed in MDCK cells. In this test egg-grown seed viruses were used.

[a] $\log_{10}$ PFU/ml less than 0.5

parental viruses. Fig. 1 shows oligonucleotide maps of two recombinants isolated in 1978 and 1980, and 66 large nucleotides were selected for comparison. It can be seen that oligonucleotide maps of both viruses were essentialy similar, detecting 80—86 per cent of the common oligonucleotides between these strains. However, in the diagram of A/sw/Ehime/1/80 9 spots (closed circles) different from the 1978-recombinant were determined (Fig. 1 a). In addition, the latter recombinant, A/sw/Kanagawa/2/78 exhibited 13 spots different from the former virus (Fig. 1 b). These results suggested that A/sw/Ehime/1/80 was derived from parental viruses different from those of A/sw/Kanagawa/2/78. In order to know the possible origin of the A/sw/Ehime/1/80

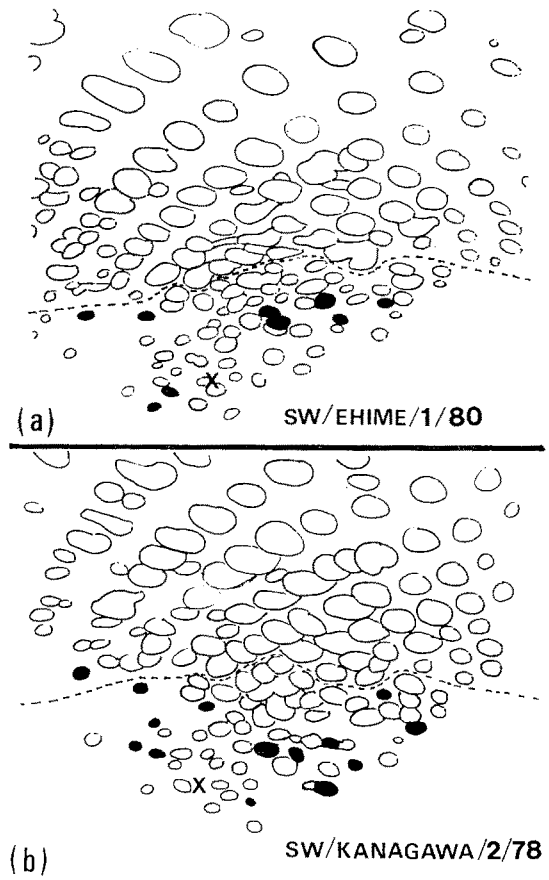

Fig. 1. Comparison of the oligonucleotide spots of the RNAs from two recombinant viruses isolated from swine in Japan in 1978 and 1980. *a* Diagram of A/sw/Ehime/1/80 (H1N2); spots indicated by closed circles were present in the A/sw/Ehime/1/80 strain but absent in a previous recombinant, A/sw/Kanagawa/2/78 (H1N2). *b* Diagram of A/sw/Kanagawa/2/78; closed circles were oligonucleotide spots present in A/sw/Kanagawa/2/78 but not in A/sw/Ehime/1/80 virus. X is the position of dye marker, xylene cyanol FF

strain, we also compared oligonucleotide maps of A/sw/Shizuoka/1/78 (H1N1) and A/Aichi/2/68 because of their antigenic and biological relatedness to the above recombinant virus. Fig. 2 shows oligonucleotide maps produced by the three viruses.

A/sw/Ehime/1/80 (Fig. 2a) and A/sw/Shizuoka/1/78 (Fig. 2c) were found to have 56 out of 66 oligonucleotide spots common (84 per cent) to these two

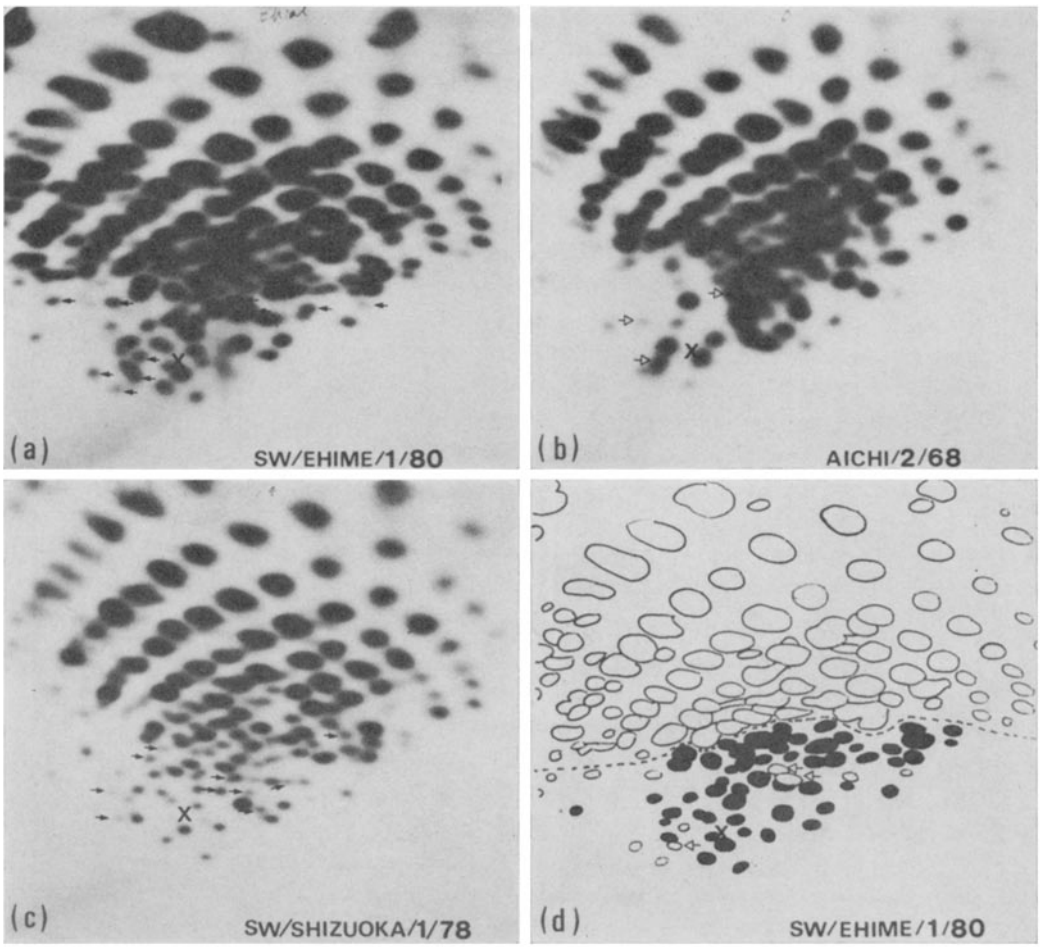

Fig. 2. Analysis of oligonucleotide maps of RNAs of swine H1N1, H1N2, and human H3N2 viruses. Spots indicated by arrows pointing to the left were detected in A/sw/Ehime/1/80 *(a)* but missing in A/sw/Shizuoka/1/78 *(c)*. The arrows directed to the right represent oligonucleotide spots present in A/sw/Shizuoka/1/78 *(c)* but missing in A/sw/Ehime/1/80. The oligonucleotides indicated by open arrows pointing to the right were derived from the neuraminidase gene of A/Aichi/2/68 *(b)*. Closed circles of the diagram *(d)* of A/sw/Ehime/1/80 were oligonucleotide spots common to A/sw/Shizuoka/1/78. Of the open circles in diagram *(d)*, 3 indicated by open arrows pointing to the left were derived from the neuraminidase of A/Aichi/2/68. X is the position of the dye marker, xylene cyanol FF

viruses. The spots indicated by arrows pointing to left (Fig. 2a) or right (Fig. 2c) were peculiarly presented in A/sw/Ehime/1/80 and A/sw/Shizuoka/1/78 strains, respectively. The minimum number of base changes between two viruses was 20. The oligonucleotide map of A/Aichi/2/68 presented in Fig. 2b was quite different from those of the above two swine isolates. The spots indicated by open arrows (Fig. 2b) represented oligonucleotides derived from neuraminidase gene of A/Aichi/2/68 (Dr. K. NAKAJIMA, personal

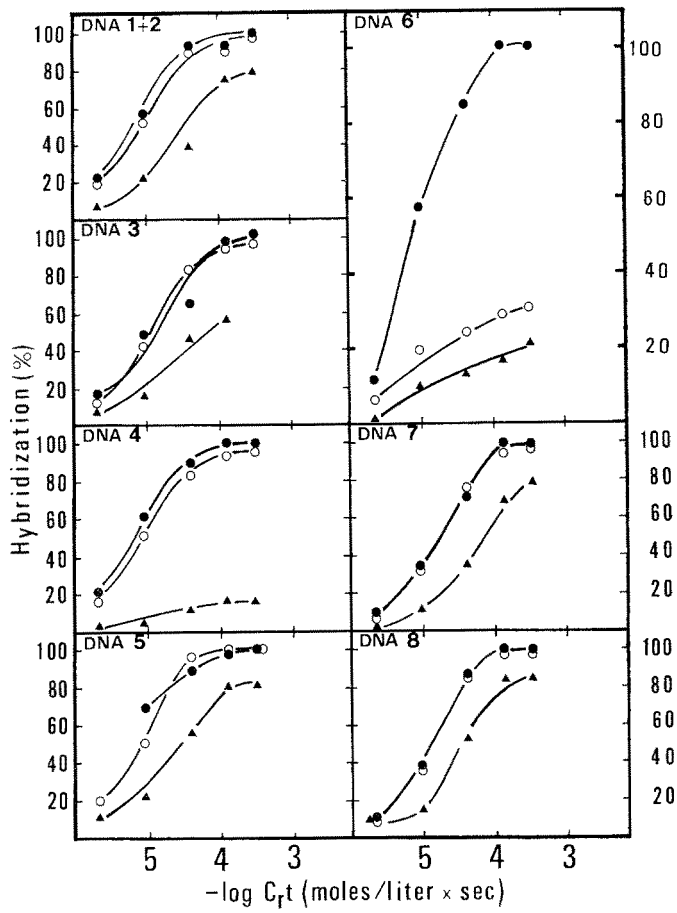

Fig. 3. Analysis of the genome composition of a recombinant, A/sw/Ehime/1/80 strain by liquid DNA-RNA hybridization. Synthesized DNAs complementary to the whole RNAs of swine (H1N1), A/sw/Kanagawa/4/78, were fractionated by 7 M-urea 4 per cent polyacrylamide gel electrophoresis (22). ^{32}P-cDNA segments were extracted from the sliced gels by reported procedures (22). Six ng of ^{32}P-cDNA segments were hybridized to increased concentrations of three whole RNAs of the A/sw/Kanagawa/4/78, A/sw/Ehime/1/80 and A/Aichi/2/68 viruses according to the methods described previously (1, 22). The rate of hybridization of cDNA with RNAs of A/sw/Kanagawa/4/78 (●———●), A/sw/Ehime/1/80 (○———○), and A/Aichi/2/68 (▲———▲) was expressed as Crt values

communication). The diagram (Fig. 2d) shows the possible derivation of oligonucleotide spots of A/sw/Ehime/1/80. These results indicated that a large proportion of spots (closed circles) were derived from A/sw/Shizuoka/1/78-like virus. Of the remaining eleven spots (open circles), 3 (indicated by open arrows) appeared to be from the neuraminidase gene of a A/Aichi/2/68-like virus.

Analysis of the Derivation of RNA Segments of a Recombinant by Liquid DNA-RNA Hybridization

To define the derivation of the RNA segments DNA-RNA hybridization was done by using six ng of ^{32}P-labelled cDNA segments for RNA of swine (H1N1) virus, A/sw/Kanagawa/4/78. The slowest moving cDNA band at the top on the 4 per cent polyacrylamide gel were collected in one fraction (P1+P2) because this band was not well resolved as described previously (22), and used in hybridization experiments. The results obtained in hybridization tests showed that cDNA segments P1+2 and 3 hybridized efficiently with the entire RNAs of a recombinant virus and a base sequence homology of nearly 100 per cent was obtained from the Crt analysis (Fig. 3). Crt was the product of RNA concentration (mol/l) and time (sec.). In contrast to this, RNAs from A/Aichi/2/68 strain exhibited a relatively low sequence homology with the above cDNA probes. When cDNA segments 4, 5, 7, and 8 were hybridized with RNAs from swine, Hong Kong and recombinant viruses, the RNAs of A/sw/Ehime/1/80 were shown to have a complete base sequence homology with the corresponding RNA of the swine (H1N1) virus, which was used for preparation of ^{32}P-cDNA. These results indicated that all RNA segments except for segment 6, of the recombinant virus were undoubtedly derived from the swine (H1N1) virus. On the contrary, only the sixth DNA segment hybridized poorly with RNA of a recombinant, and this low sequence homology was similar to that of A/Aichi/2/68 virus. Evidently, A/sw/Ehime/1/80 is a recombinant virus and only the neuraminidase gene was from A/Aichi/2/68.

Discussion

A previous report (22) and the present antigenic analysis of the hemagglutinin subunit of H1N1 (Hsw1N1) swine isolates revealed that two influenza virus variants have been prevalent in the swine population of Japan. The major variant group reacted with all monoclonal antibodies to A/NJ/8/76, whereas the minor variant group failed to react with 2 of them. Although the A/sw/Kanagawa/2/78 recombinant virus contained a hemagglutinin antigen identical to that of the former variant (22), the A/sw/Ehime/1/80 strain was shown to have a hemagglutinin related closely to the latter minor variant group. It is of interest to know that two recombinant

viruses clearly reflect epizootiological aspects of swine influenza in Japan based on virological studies. Since the appearance of the Hong Kong (H3N2) virus in man, numerous contemporary strains which had been prevalent in the human population have been isolated from swine (10, 20, 21, 29, 34, 36) and the isolation of two recombinants from pigs in Japan indicated that the swine was a potential reservoir for human influenza. Recent antigenic and genetic studies on different H3N2 swine isolates described that Hong Kong influenza virus-like strains similar to A/Aichi/2/68 have been maintained in swine until 1976 (20, 36). Our detailed investigation of the antigenic structure of the neuraminidase of different H3N2 swine isolates with monoclonal antibodies supports the above evidence. In addition, a swine recombinant virus isolated in 1980 was found to possess a neuraminidase antigenically very similar to that of these earliest Hong Kong (H3N2)-like viruses, suggesting that an A/Aichi/2/68-like virus which had already disappeared from the human population 12 years before have been circulating in the swine population.

Unlike human viruses, influenza viruses originating from avian species are able to replicate at stringent temperatures such as 42° C (9, 18, 23). The present studies first demonstrated that swine isolates have a capability of reproducing at 42° C, which is similar to avian influenza viruses. A swine recombinant, A/sw/Kanagawa/2/78 (H1N2) had its possible H1N1 parental viruses are capable of reproducing in MDCK cells maintained at 42° C, and their efficiency of plaque formation is very high. Our previous work revealed that 7 RNA segments of this recombinant are derived from swine (H1N1) virus and the remainder, coding for neuraminidase is from human A/Kumamoto/22/76 (H3N2) (A/Victoria/3/75-like strain) (22). However, the latter human H3N2 virus does not grow at 42° C, so that the property of this recombinant to grow at a high temperature, therefore, comes from the A/sw/Kanagawa/4/78 (H1N1) virus. Recent information suggests that transmission of human viruses to swine occurs frequently as described above. All avian influenza viruses examined can replicate to high titers in pigs (12), suggesting that swine is one of the important reservoir where dual infection with avian and human influenza viruses could occur in nature.

The above studies also revealed that A/sw/Ehime/1/80 virus is generated by genetic recombination between parental viruses different from those of the A/sw/Kanagawa/2/78 recombinant, showing that the former recombinant virus isolated in 1980 is not able to grow at 42° C. Hybridization studies indicated that A/sw/Kanagawa/4/78 (H1N1) which can replicate at a high temperature, and A/sw/Ehime/1/80 were found to contain seven very similar RNA segments. With the exception of segment 6, these high base sequence homologies of all genes between A/sw/Kanagawa/4/78 (H1N1) and A/sw/Ehime/1/80 based on molecular hybridization tests may imply that the different biological properties between both viruses depend on the

restrictive changes of some of the structural genes, but definite conclusion must await further studies.

Acknowledgements

The authors would particularly like to thank Drs. R. G. Webster and V. S. Hinshaw, St. Jude Children's Research Hospital, U.S.A., for providing splendid antiserum and monoclonal antibodies to hemagglutinin and neuraminidase.

We gratefully thank Dr. Dennis J. Alexander, Central Veterinary Laboratory, Surrey, England, for his critical reading of the manuscript. This work was supported by research grants from the Science and Technology Agency, and Ministry of Education, Japan.

References

1. Avvedimento, V. E., Vogel, G., Yamada, Y., Maizel, J. V., Jr., Pastan, I., Crombrugghe, B.: Correlation between splicing sites within an intron and their sequence complementary with U1 RNA. Cell **21**, 689—696 (1980).
2. Aymard-Henry, M., Coleman, M. T., Doudle, W. R., Laver, W. G., Schild, G. C., Webster, R. G.: Influenza virus neuraminidase and neuraminidase inhibition test procedures. Bull. WHO **48**, 199—202 (1973).
3. Billeter, M. A., Parson, J. T., Coffin, J. M.: The nucleotide sequence complexity of avian tumor virus RNA. Proc. Nat. Acad. Sci. U.S.A. **71**, 3560—3564 (1974).
4. Cambell, C. H., Webster, R. G., Breese, S. S., Jr.: Fowl plaque virus from man. J. Infect. Dis. **122**, 513—516 (1970).
5. Davis, A. R., Nayak, D. P., Ueda, M., Hiti, A. L., Doubenko, D.: Expression of antigenic determinants of the hemagglutinin gene of a human influenza virus in Escherichia coli. Proc. Nat. Acad. Sci. U.S.A. **78**, 5376—5380 (1981).
6. Desselberger, U., Nakajima, K., Alfino, P., Pederson, F. S., Haselstine, W. A., Palese, P.: Biochemical evidence that "new" influenza virus strains in nature may arise by recombination (reassortment). Proc. Nat. Acad. Sci. U.S.A. **75**, 3341—3345 (1978).
7. Fang, R., Jou, W. M., Huylebroeck, D., Devos, R., Fiers, W.: Complete structure of A/duck/Ukraine/63 influenza hemagglutinin gene: Animal virus as progenitor of human H3 Hong Kong 1968 influenza hemagglutinin. Cell **25**, 315—323 (1981).
8. Francis, T., Jr., Magill, T. P.: The incidence of neutralizing antibodies for human influenza virus in the serum of human individuals of different ages. J. Exp. Med. **63**, 655—668 (1936).
9. Ghendon, Y. Z., Klimov, A. I., Ginsburg, V. P.: Studies of a recombinant which inherited the hemagglutinin from the human influenza virus A/Hong Kong/1/68 (H3N2) and other genes from influenza virus A/duck/Ukraine/1/63 (H3N2). J. gen. Virol. **65**, 165—172 (1984).
10. Hinshaw, V. S., Bean, W. J., Jr., Webster, R. G., Easterday, B. C.: The prevalence of influenza viruses from man and swine. Virology **84**, 51—62 (1978).
11. Hinshaw, V. S., Webster, R. G., Rodrinquez, R. J.: Influenza A viruses: Combination of hemagglutinin and neuraminidase subtypes isolated from animals and other sources. Arch. Virol. **67**, 191—202 (1981).
12. Hinshaw, V. S., Webster, R. G., Easterday, B. C., Bean, W. J., Jr.: Replication of avian influenza A viruses in mammals. Infect. Immun. **34**, 354—361 (1981).
13. Hinshaw, V. S., Air, G. M., Gibbs, A. J., Graves, L., Prescott, B., Karunakaran, D.: Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. J. Virol. **42**, 865—872 (1982).

14. Koen, J. S.: A practical method for field diagnosis of swine diseases. Am. J. Vet. Med. **14**, 468—470 (1919).
15. Kundin, W. D.: Hong Kong A2 influenza virus infection among swine during a human epidemic in Taiwan. Nature (London) **228**, 957—958 (1970).
16. Laver, W. G., Webster, R. G.: Studies on the origin of pandemic influenza. III. Evidence implicating ducks and equine influenza viruses as possible progenitors of the Hong Kong strains of human influenza. Virology **51**, 383—391 (1973).
17. Lvov, D. K., Zhdanov, V. M.: Persistence of genes of epidemiological influenza viruses in natural populations in the U.S.S.R. Med. Biol. **61**, 83—91 (1983).
18. Murphy, B. R., Hinshaw, V. S., Sly, D. L., London, W. T., Hosier, N. T., Wood, F. T., Webster, R. G., Channock, R. M.: Virulence of avian influenza A viruses for squirrel monkeys. Infect. Immun. **37**, 1119—1126 (1982).
19. Nakajima, K., Desselberger, U., Palese, P.: Recent human influenza A (H1N1) viruses are closely related to strains isolated in 1950. Nature (London) **274**, 334—339 (1978).
20. Nakajima, K., Nakajima, S., Shortridge, K. F., Kendal, A. P.: Further genetic evidence for maintenance of early Hong Kong-like influenza A (H3N2) strains in swine until 1976. Virology **116**, 562—572 (1982).
21. Nerome, K., Ishida, M., Nakayama, M., Oya, A., Kanai, C., Suwicha, K.: Antigenic and genetic analysis of A/Hong Kong (H3N2) influenza viruses isolated from swine and man. J. gen. Virol. **56**, 441—445 (1981).
22. Nerome, K., Sakamoto, S., Yano, N., Yamamoto, T., Kobayashi, S., Webster, R. G., Oya, A.: Antigenic characteristics and genome composition of a naturally occurring recombinant influenza virus isolated from a pig in Japan. J. gen. Virol. **64**, 2611—2620 (1983).
23. Nerome, K., Yoshioka, Y., Torres, C. A., Oya, A., Bachmann, P., Ottis, K., Webster, R. G.: Persistence of Q strains of H2N2 influenza virus in avian species: Antigenic, biological and genetic analyses of avian and human H2N2 viruses. Arch. Virol. **81**, 239—250 (1984).
24. Palese, P., Schulman, J. L.: Mapping of the influenza virus genome: Identification of the hemagglutinin and the neuraminidase genes. Proc. Nat. Acad. Sci. U.S.A. **73**, 2142—2146 (1976).
25. Pederson, F. S., Haselstine, W. A.: A micromethod for detailed characterization of high molecular weight RNA. In: Grossman, L., Moldave, K. (eds.), Method in Enzymology, 680—687. Academic Press 1980.
26. Pensaet, M. M., Ottis, K., Vandeputte, J., Kaplan, M. M., Bachmann, P. A.: Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man. Bull. WHO **59**, 75—78 (1981).
27. Richey, M. B., Palese, P., Schulman, J. L.: Mapping of the influenza virus genome. III. Identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein. J. Virol. **20**, 307—313 (1976).
28. Russ, G., Vaerckova, E., Styk, B.: Steric effect in the reaction of influenza virus neuraminidase with antibodies. Acta Virol. **18**, 229—306 (1974).
29. Schild, G. C., Brand, C. M., Harkness, J. W., Lamont, P. H.: Studies on relationships between human and porcine influenza. 2. Immunological comparisons of human A/Hong Kong/68 virus with influenza A viruses of porcine origin. Bull. WHO **46**, 721—728 (1972).
30. Scholtissek, C., Rohde, W., von Hoynigen, V., Rott, R.: On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology **87**, 13—20 (1978).
31. Shope, R. E.: Swine influenza. 1. Experimental transmission and pathology. J. Exp. Med. **54**, 349—360 (1931a).

32. SHOPE, R. E.: Swine influenza. III. Filtration experiments and etiology. J. Exp. Med. **54**, 373—380 (1931b).
33. SHOPE, R. E.: The incidence of neutralizing antibodies for swine influenza virus in the sera of human beings of different ages. J. Exp. Med. **63**, 669—684 (1936).
34. SHORTRIDGE, K. F., WEBSTER, R. G., BUTTERFIELD, W. K., CAMPBELL, C. H.: Persistence of Hong Kong influenza virus variants in pigs. Science **196**, 1454—1455 (1976).
35. SHORTRIDGE, K. F., WEBSTER, R. G.: Geographical distribution of swine (Hsw1N1) and Hong Kong (H3N2) influenza virus variants in pigs in South East Asia. Intervirol. **11**, 9—15 (1979).
36. SHORTRIDGE. K,, F., CHERRY, A., KENDAL, A. P.: Further studies of the antigenic properties of H3N2 strains of influenza A isolated from swine in South East Asia. J. gen. Virol. **44**, 251—254 (1979).
37. STUART-HARRIS, C. H., SCHILD, G. C.: The epidemiology of influenza virus. 112—113. In: STUART-HARRIS, C. H., SCHILD, G. C. (eds.), Influenza, The Viruses and the Diseases, 112—113. Edward Arnold 1976.
38. YASUHARA, H., HIRAHARA, T., NAKAI, M., SASAKI, N., KATO, J., WATANABE, T., MORIKAWA, M.: Further isolation of a recombinant virus (H1N2, formerly Hsw1N2) from a pig in Japan in 1980. Microbiol. Immunol. **27**, 43—50 (1983).

Authors' address: Dr. K. NEROME, Department of Virology and Rickettsiology, National Institute of Health, Shinagawa-ku, Tokyo 141, Japan.

Received February 28, 1985