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Difference in growth behavior of human, swine, equine, and avian influenza viruses at a high temperature

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Summary. Growth characteristics of a wide range of influenza A viruses from different mammals and bird species were examined in an established line of canine kidney (MDCK) cells at an ordinary (37 °C) and a high temperature (42 °C). Although all viruses employed in the present study possessed a capability of replicating at 37 °C, virus growth at 42 °C showed considerable variation and reflected differences in the natural hosts of the isolates. All reference strains and isolates from bird species grew well in the MDCK cells maintained at 42 °C, but human viruses did not, showing an asymmetrical growth behavior.

In contrast to this, growth of swine and equine viruses showed growth characteristics intermediate between human and avian viruses. Of the two swine viruses examined, replication of one strain occured equally well at both temperatures and another failed to grow at 42 °C. Similarly, two of the three equine viruses tested belonging to H3N8 antigenic subtypes grew at 42 °C. However, the results obtained from comparison of plaque sizes and growth curves indicated that the replication of the above swine and equine viruses was restricted under a stringent temperature when compared to avian viruses. The detailed analysis of cloned viruses revealed that some of the swine and equine viruses contained two variants which are readily distinguished by growth behavior at 42 °C. Genome analysis of parental and virus clones by oligonucleotide mapping and migration profiles of RNA segments did not detect any differences among the above variants exhibiting the asymmetrical growth characteristics at 42 °C.

Introduction

Influenza A viruses are divided into 13 hemagglutinin and 9 neuraminidase subtypes according to the revised nomenclature system recommended by an Expert Committee of the World Health Organization [32]. The detailed investigation of antigenic and genome structures of the earliest Asian (H2N2) and Hong Kong (H3N2) viruses strongly suggest that new types of influenza A

viruses which suddenly appear in man may be associated with avian influenza reservoirs [8, 20, 22]. An intensive study on surveillance of influenza viruses indicated that a great number of the viruses possessing all hemagglutinin (HA) and neuraminidase (NA) subtypes with different combinations have been circulating in birds, especially feral and domestic ducks [6, 9, 22, 25, 26, 30]. Coupled with the evidence that HA and NA antigens of all mammalian viruses which have been isolated until now are related immunologically to avian viruses [22], the bird world is considered as a key to understand natural ecology of influenza virus. Since the genomic structure of gene constellation controlling host specificity is not well understood, it is of fundamental importance to accumulate any evidence recognizing the host differences. For this reason, recent reports concerning tissue tropism would appear worthy of evaluation in conferring a clue for solving the mode of circulation of influenza virus in birds and different species of lower animals [1, 5, 24, 31]. In addition, the evidence that avian virus can grow at a high temperature [3, 11, 15, 21] led us to compare in details the replication of a total of 30 viruses from four natural hosts at 42 °C. In the present communication we describe growth characteristics of avian, equine, swine and human influenza A viruses and one strain of influenza B virus at 37 °C and 42 °C.

Materials and methods

Viruses

The following influenza A viruses from birds, horses, pigs and humans were employed in the present study: A / turkey / England / 63(H7N7), A / chicken / Germany "N" / 49(H10N7), A / duck / England / 56(H11N6), A / duck / Czech / 56(H4N6), A / tern / S. Africa / 61(H5N3), A / turkey / Mass / 65(H6N2), A / duck / Ukraine / 1 / 63(H3N8), A / turkey / Ontario / 6118 / 68 (H8N4), A / turkey / Wisconsin / 66 (H9N2), eight exotic bird isolates from Japan shown in Table 1, A / USSR / 92 / 77(H1N1), A / PR / 8 / 34(H1N1), A / NWS / 33(H1N1), A / Jap / 307 / 57(H2N2), A / Aichi / 2 / 68(H3N2), B / Singapore / 222 / 79, and A / NJ / 8 / 76(H1N1), A / equine / Prague / 1 / 56(H7N7), A / equine / Miami / 1 / 63(H3N8), A / equine / Niigata / 1 / 71(H3N8), A / swine / Iowa / 15 / 30(H1N1), and A / swine / Shimane / 1 / 78(H1N1). For reference, the former names of antigenic subtypes of reference strains and isolates are given in Table 1. All the viruses used in the tests were grown in the allantoic cavity of 11-day-old embryonated hens' eggs.

Growth tests

Monolayers of MDCK cells prepared in 60 mm Falcon's petri dishes were infected with a MOI ranging from 0.2 to 0.02 plaque-forming units (PFU) of the seed virus per a cell, and the viruses were allowed to adsorb for 30 min at 35 °C. The unadsorbed virus was then removed by washing twice with phosphate buffered saline (PBS, pH 7.2) and the infected cells were incubated under 5 per cent CO_2 at the indicated temperature after adding maintenance medium containing 5 μg/ml of trypsin. For examination of the virus growth, aliquots of culture fluids were removed daily, and their HA activity and plaque infectivity were measured as described in previous report [27]. Virus cloning was initiated by "picking" plaques produced in MDCK cells and subsequent plaque purification was undertaken by repeating the above procedures.

Biological assays

HA titration and hemagglutination-inhibition (HI) tests were performed using 0.5% chicken red blood cells and microtiterplates under the room temperature. Antisera used in the HI tests were treated with recepter-destroying enzyme (RDE) and, before use, non-specific hemagglutinin was removed by adsorbing with chicken red blood cells.

RNA analysis

Migration profiles of RNA segments of equine and swine influenza viruses were examined as described [17]. Briefly, two confluent dishes (64 cm^2) of MDCK cells per a strain were prepared and infected with the viruses at a MOI of 0.03–0.7 PFU/cell, and approx. 225 μCi of ^{32}P-phosphoric acid was added to each dish containing maintenance medium without phosphoric acid. Before and after infection, cells were washed well with Earle's balanced salt solution free from phosphoric acid and viruses were grown at 35 °C for 3 days. When infected cells showed CPE-values more than plus 3, the ^{32}P-labelled viruses were harvested and purified by discontinuous sucrose gradient centrification according to the methods described by Ueda and Tobita [28], and RNA was extracted as described previously [17]. RNA segments were fractionated on 2.8% polyacrylamide gel electrophoresis containing 6M Urea [17, 28]. For oligonucleotide mapping of whole viral RNA's, egg-grown viruses were purified by the method described by Nerome [13, 14].

Results

Growth comparison of avian and mammalian viruses

Although the previous study revealed that avian and human influenza A viruses show different characteristics of replication at high temperatures such as 42 °C, this was further investigated using a panel of reference strains and isolates of human, swine, equine and avian viruses. As shown in Table 1, virus growth was first screened under the condition of multisteps of growth cycle by examining HA activity produced in the culture fluids. The results indicated that, without exception, all reference strains and isolates from birds grew at 42 °C as well as at 37 °C, but HA activity of human viruses was detected only at 37 °C. In contrast to the above viruses, equine and swine viruses showed more complicated growth characteristics at the higher temperature. Of four equine strains 2 belonging to H3N8 subtype were found to grow at 42 °C, reaching HA titer of 32–64 on the 3rd day post infection. However, virus yields obtained at 37 °C were considerably higher than those at 42 °C, implying replication of the viruses was somewhat affected under the condition of stringent temperature. It was also of interest to observe that one swine virus was capable reproducing at the higher temperature and its titer was similar to that at 37 °C. The present study revealed that swine and equine virus possessed growth characteristics similar in part to both the human and avian viruses. Despite these results, it is not clear if progeny viruses produced at 42 °C possess the antigenic characteristic peculiar to swine or equine viruses. In order to determine this, antigenic analysis of the viruses yielded at 37 °C and 42 °C was performed by HI tests with post-infection ferret antisera against A / eq / Miami / 1 / 63(H3N8), A / eq / Tokyo / 3 / 71(H3N8), and A / NJ / 8 / 76(H1N1) viruses. The results obtained in cross-HI tests indicated that two equine strains, A / eq / Miami / 1 / 63(H3N8)

Table 1. Growth comparison of avian and mammalian influenza viruses at 37 °C and 42 °C

Origin	Virus	Antigenic subtypes		HA titer in the culture fluids					
				Days					
				37 °C			42 °C		
				1	2	3	1	2	3
Avian	A/duck/Ukraine/1/63	H3N8	(Hav7Neq2)[a]	64	128	128	128	128	128
	A/duck/Czech/56	H4N6	(Hav4Nav1)	—[b]	64	64	—	64	64
	A/tern/S. Africa/61	H5N3	(Hav5Nav2)	—	64	64	—	64	64
	A/turkey/Mass/65	H6N2	(Hav6N2)	—	4	4	—	4	8
	A/turkey/England/63	H7N7	(Hav1Nav3)	128	128	128	32	64	64
	A/turkey/Ontario/6118/68	H8N4	(Hav8Nav4)	2	32	32	2	32	32
	A/turkey/Wisconsin/66	H9N2	(Hav9N2)	—	64	64	—	64	64
	A/chicken/Germany "N"/49	H10N7	(Hav2Neq1)	128	128	128	—	64	64
	A/duck/England/56	H11N6	(Hav3Nav1)	—	128	128	—	8	64
	A/mynah/Tokyo/7/76	H3N8	(Hav7Neq2)	32	32	32	32	64	64
	A/mynah/Tokyo/9/76	H3N8	(Hav7Neq2)	2	64	64	8	64	128
	A/mynah/Tokyo/11/76	H3N8	(Hav7Neq2)	64	64	128	64	128	128
	A/banded parakeet/Tokyo/67/76	H3N8	(Hav7Neq2)	4	64	128	16	64	64

	A/mynah/Tokyo/15/76	H4N8	(Hav4Neq2)	16	32	32	16	32	32
	A/mynah/Tokyo/23/76	H4N8	(Hav4Neq2)	8	32	64	32	32	64
	A/mynah/Tokyo/87/76	H4N8	(Hav4Neq2)	16	32	32	16	32	32
	A/mynah/91/76	H4N8	(Hav4Neq2)	4	32	64	16	32	32
	A/eq/Niigata/1/71	H3N8	(Heq2Neq2)	2	64	128	—	—	—
Equine	A/eq/Tokyo/3/71	H3N8	(Heq2Neq2)	16	128	64	—	8	32
	A/eq/Miami/1/63	H3N8	(Heq2Neq2)	32	256	128	—	64	64
	A/eq/Prague/1/56	H7N7	(Heq1Neq1)	2	2	4	—	—	—
Swine	A/sw/Iowa/15/30	H1N1	(Hsw1N1)	8	128	128	—	—	—
	A/sw/Shimane/1/78	H1N1	(Hsw1N1)	4	64	32	—	32	64
	A/USSR/92/77	H1N1		16	64	128	—	—	—
	A/PR/8/34	H1N1	(H0N1)	16	64	256	—	—	—
	A/NWS/33	H1N1		64	—	—	—	—	—
Human	A/NJ/8/76	H1N1	(Hsw1N1)	4	256	256	—	—	—
	A/Jap/307/57	H2N2		—	32	64	—	—	—
	A/Aichi/2/68	H3N2		8	32	64	—	—	—
	B/Singapore/222/79	B		32	128	256	—	—	—

[a] Subtype in parenthesis represents the former nomenclature
[b] Less than 2

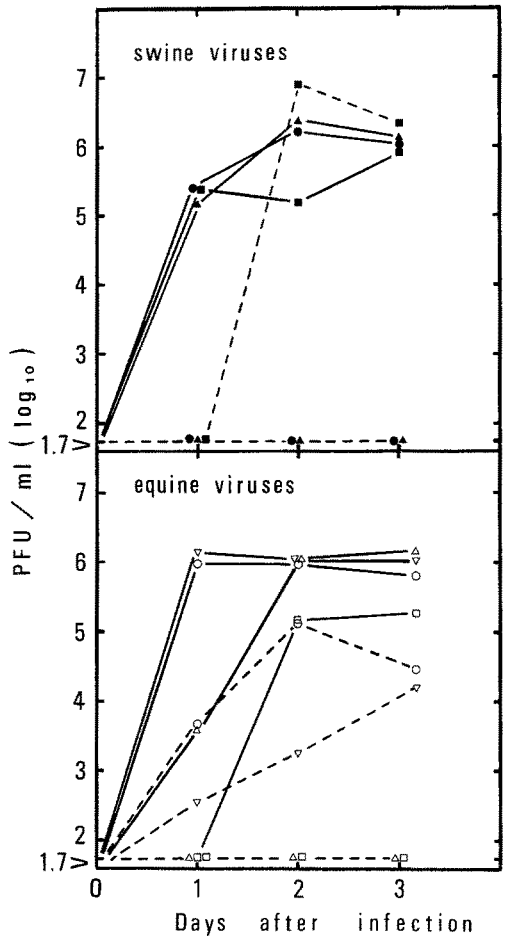

Fig. 1. Replication of swine and equine influenza viruses in MDCK cells maintained at 37 °C (———) and 42 °C (– – –). Portion of the culture fluids was removed daily and titrated for plaque-infectivity. Closed symbols represent swine strains (● Iowa/ 15 / 30; ■ Shimane / 1 / 78) and a human strain, similar antigenically to swine strains (▲ NJ / 8 / 76) and open ones represent equine strains (○ Miami / 1 / 63; ▽ Tokyo / 3 / 71; □ Prague / 1 / 56; △ Niigata / 1 / 71)

and A / eq / Tokyo / 3 / 71(H3N8), and one swine strain, A / sw / Shimane / 1 / 78 possessed undoubtedly antigenic properties identical to those of original viruses, indicating no contamination with avian-like viruses (data not shown). Since some of the swine and equine viruses appeared to grow at the higher temperature, virus replication was monitored in detail by titrating the infectious virus in culture fluids taken daily. Figure 1 shows growth curves of swine and equine viruses obtained in multicycles of growth experiments. Of two strains examined A / sw / Shimane / 1 / 78 grew well at 42 °C and the levels of plaque-infectivity increased markedly during the first 2 days, with plaque-titers of $10^{7.0}$ at days 2 post infection, and tended to decline thereafter. Similarety, of 4 equine strains tested A / eq / Miami / 1 / 63 and A / eq / Tokyo / 3 / 71 strains have

the ability to replicate at 42 °C, although the viruses were produced at slightly lower titers, with the peak at day 2 after infection.

Plating efficiency of parental and cloned viruses

The findings on the growth behavior of swine and equine viruses similar to the avian virus led to further investigation to determine whether these viruses can grow at a high temperature through the mechanism by which adaptation occurred during incubation or if the co-presence of two kinds of phenotypic variants in a virus population results in selection of the variants which are able to grow at a high temperature. In order to determine this, two equine and one swine viruses were plaque-titrated in parallel at 37 °C and 42 °C. Table 2 shows the virus infectivity yielded by the three viruses. Of two equine viruses examined, Miami / 1 / 63 strain produced plaques with high efficiency at both temperatures, and the mean titers at 42 °C were $10^{7.6}$ PFU/ml, as compared to $10^{8.3}$ PFU/ml at 37 °C. In contrast, another equine strain, Tokyo / 3 / 71 showed significant reduction in plaque infectivity at 42 °C in comparison with its yield at 37 °C and the ratio of plating efficiency decreased to 0.009. In addition, this tendency was observed in the swine virus; plaque titers at 37 °C and 42 °C were $10^{7.2}$ and $10^{6.1}$ PFU/ml, respectively. The differences in plating efficiency reflected on the plaque sizes formed at both temperatures (data not shown), indicating the suppression of replication of these mammalian viruses by the elevated temperature. It was of interest to know that Miami / 1 / 63 strain formed somewhat larger plaques even at 42 °C when compared to the other viruses, which is in agreement with higher plating efficiency of this virus. It is noticeable that plaques produced by an avian isolate, similar antigenically to human H2N2 viruses, were almost equal in size at both temperatures (data not shown).

Table 2. Parallel plaque titration of equine and swine influenza viruses in MDCK cells maintained at 37 °C and 42 °C

Strain	PFU/ml		ratio (42 °C/37 °C)
	37 °C	42 °C	
A/eq/Miami/1/63	3.95×10^8	6.25×10^7	0.158
A/eq/Tokyo/3/71	2.10×10^8	1.91×10^6	0.009
A/sw/Shimane/1/78	2.02×10^7	1.62×10^6	0.080

Plaque titration was done in MDCK cells with trypsin [27]

Growth characteristics of parental and cloned viruses at different temperatures

The parallel plaque titration tests suggest that the above 3 viruses derived from two different mammals may contain two kinds of virus variants of which one

Table 3. Efficiency of plaque formation of equine and swine influenza viruses in MDCK cells under the conditions of elevated temperatures

Viruses	Plaque titer (log_{10} PFU)					Shut-off temperature
	35 °C	37 °C	39 °C	41 °C	42 °C	
A/eq/Miami/1/63	8.65	8.86	8.60	8.01	7.15	>42 °C
M37-2[a]	8.85	8.62	6.70	<3.40	<3.40	41 °C
M42-3[a]	8.65	8.61	8.46	8.27	7.79	>42 °C
A/eq/Tokyo/3/71	8.29	8.30	8.06	7.40	5.35	>42 °C
A/eq/Prague/1/56	6.78	6.39	3.40	<1.40	<1.40	39 °C
A/eq/Niigata/1/71	7.74	7.44	5.92	<2.40	<2.40	41 °C
A/sw/Shimane/1/78	7.83	7.94	7.91	7.45	6.61	>42 °C
S37-4[a]	7.57	7.46	7.06	<2.40	<2.40	41 °C
S42-3[a]	7.60	7.56	7.48	7.10	6.67	>42 °C
A/sw/Iowa/15	8.59	8.57	8.08	<2.40	<2.40	41 °C

A second agar overlay was done on the 3 days after infection and plaques were counted on the following day

[a] Cloned viruses from parental equine (Miami/1/63) and swine (Shimane/1/78), respectively

replicates at 42 °C but the other does not. In order to elucidate this hypothesis, eq / Miami / 1 / 63 and sw / Shimane / 1 / 78 viruses were plaqued on MDCK cells and the plaques picked were purified through subsequent plaque formation. cloned viruses derived from eq / Miami / 1 / 63 were tested for growing ability at both temperatures, 2 of the 24 were found to replicate at 42 °C and the remaining 22 failed to produce plaques under the stringent condition of temperature. The results obtained here revealed that the equine virus variant possessing ability of replicating at high temperature accounted for about a tenth of the total population, in accord with the high plating efficiency shown in Table 2.

We could also select two types of swine virus variants which exhibited asymmetrical growth behavior at 42 °C. As shown in Table 3, plaquing efficiencies of wild and cloned viruses were again examined in MDCK cells maintained at different temperatures ranging from 35 to 42 °C. Two equine (Prague / 1 / 56, Niigata / 1 / 71) and one swine (Iowa / 15 / 30) strains failed to induce plaques even at 41 °C and the shut-off temperature appeared to be 39 °C based on the lowest temperature at which there was 10^4 or greater decrease in plaque titers.

It can also be seen that two clones (M42-3, S42-3) produced plaques with high efficiency at 42 °C, whereas the remaining two (M37-2, S37-4) did not produce plaques at a temperature higher than 41 °C. Although the former viruses tended to reduce virus yield with increment of temperature, their plaquing efficiency was still higher compared to their parental virus.

Genome analysis of the parental and cloned viruses

Finding of co-existence and cloning of two variants which show different growth behavior at 42 °C suggest the need for further characterization of swine and equine viruses if the difference in growth ability of these viruses was due to the introduction of RNA segments from avian virus or to a very limited change of genome structure such as point mutation. For this purpose, RNAs were extracted from six egg-grown viruses and electrophoresed through a 2.8% polyacrylamide gel containing 6M urea. Figure 2 shows migration profiles of

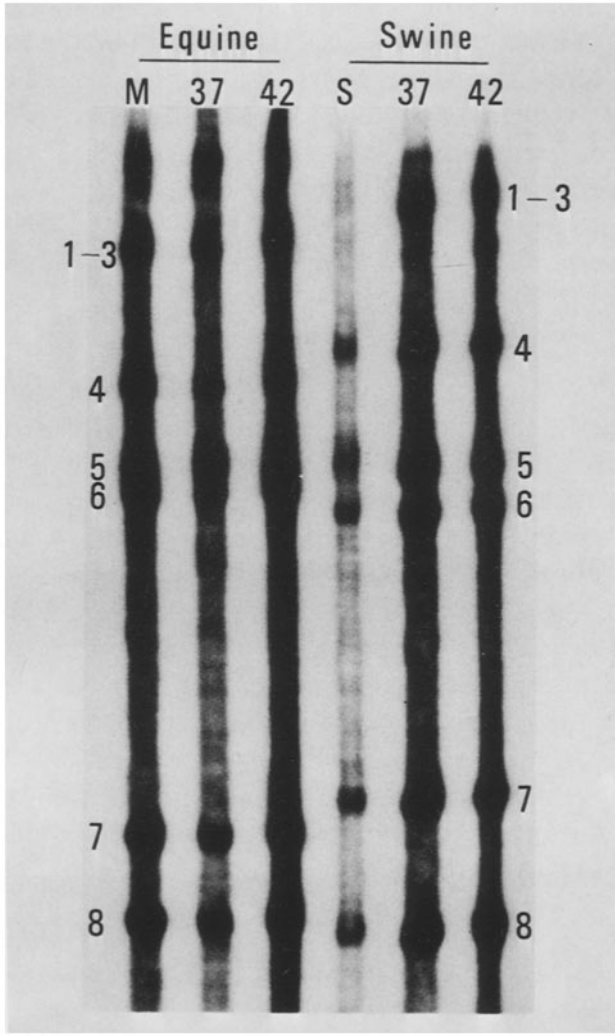

Fig. 2. Comparison of migration profile of RNA segments 1 to 8 of swine and equine influenza viruses. RNAs were in vivo-labelled with ^{32}P-orthophosphate in 90 mm dishes and extracted RNA segments were separated by electrophoresis on a 28 cm polyacrylamide slab gel. *M* Miami / 1 / 63, *37* cloned virus from Miami / 1 / 63, *42* cloned virus from Miami / 1 / 63; *S* Shimane / 1 / 78, *37* cloned virus from Shimane / 1 / 78, *42* cloned virus from Shimane / 1 / 78

whole RNA segments of each of the viruses examined. Only 7 of the eight segments were detected, probably due to co-migration of two kinds of the slowest moving polymerase genes. The RNA patterns of sw / Shimane / 1 / 78 and its cloned viruses (S37-4, S42-3) were indistinguishable, while they were definitely different from those of equine viruses. Similarly, it can be seen that 7 RNA segments of eq / Miami / 1 / 63 migrated in the same position as two cloned viruses (M37-2, M42-3) derived from the former. These results suggest that the two kinds of virus variant present in the swine or equine virus populations possess RNA genomes similar to each other.

In order to determine this, three equine viruses were selected and their RNAs were further compared by the oligonucleotide mapping method. The results presented in Fig. 3 show the oligonucleotide maps of whole RNAs of parental Miami / 1 / 63, M37-4 and cloned M42-3 viruses. The latter two cloned viruses were quite identical to each other in their migration patterns of oligonucleotides, while they were different only at one spot indicated (Fig. 3b, c) by arrow from the parental virus (Fig. 3a). This finding increased the possibility that asymmetrical difference in growth behavior of M37-4 and M42-3 viruses at 42 °C may be due to very restricted changes of the genome structure.

Discussion

All influenza A viruses circulating in different mammals from human beings to whales appear to share a high degree of antigenic relatedness with avian influenza virus isolates [5, 8, 25]. The above evidence revealed by WHO surveillance programs of influenza suggests difficulty in distinguishing mammalian from avian viruses on the basis of antigenic subtypes. The majority of epidemiological or epizootiological studies have been aimed chiefly at promotion of understanding of a mode of prevalence of human influenza. Despite numerous attempts, the mechanisms by which a new influenza viruses suddenly appear in man or break through the barrier between natural hosts are not well understood, indicating the importance of constant accumulation of knowledge relevant to host specificity. In view of what had preceded, the evidence that growth characteristics of a human virus in bovine kidney (MDBK) cells is controlled by the neuraminidase gene [24] appears to be of considerable importance. Recent studies have also revealed that loss or appearance of pathogenicity of an in-

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Fig. 3. Oligonucleotide mapping analysis of the RNAs of equine parental and cloned viruses. Egg-grown viruses were concentrated by centrifugation at 44,000 g for 90 min in Beckman 21 rotor and purified in linear sucrose gradients, 10 to 50% (w/w), at 64,000 g for 120 min using a sw27 rotor. Extracted RNAs were digested with ribonuclease T1 and 5′-end-labelled with [^{32}P] ATP in the presence of polynucleotide kinase according to the method described previously [14, 15]. Two dimensional separation of labelled nucleotides was done as described [2, 15]. An arrow indicates the spot present in parental virus (**a**), but absent in two cloned viruses (**b, c**). *X B* Positions of the dye markers xylene cyanol FF and bromophenol blue

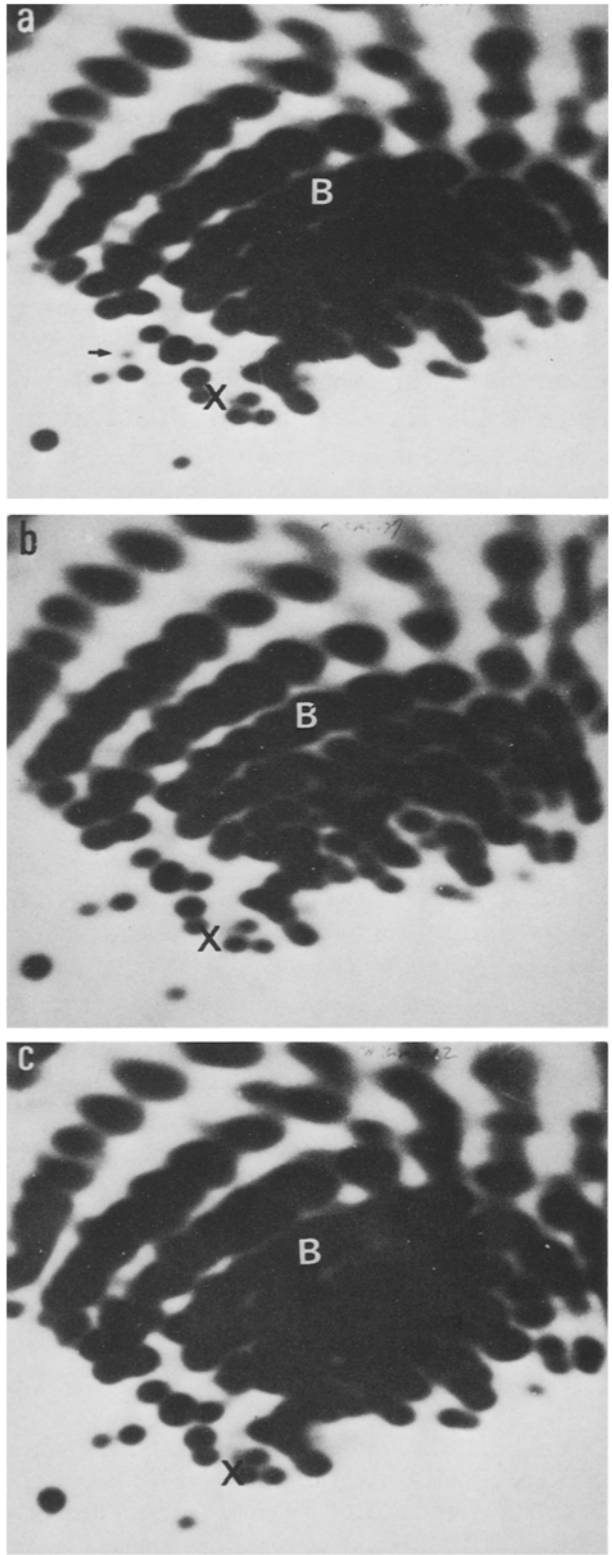

Fig. 3

fluenza virus for mice or chickens are closely related to occurrence of replacement of a few RNA segments [7, 12, 16, 23].

Our study dealing with a large number of avian and mammalian viruses confirmed an earlier report that avian influenza viruses grew at 42 °C but human viruses did not [11]. One conclusion from the two studies is that human influenza virus may be easily distinguishable from avian by determination of growth at the high temperature. In contrast, some strains of swine and equine viruses were found to possess the ability to replicate at 42 °C, showing intermediate characteristics between avian and human influenza viruses. Coupled with this evidence, it is of interest that avian influenza viruses have been reported to replicate to high titers in pigs and that the progeny virus could be transmitted to other contact pigs [4, 18]. These results strongly suggest that an avian virus, or its genes, can be conserved in a pig population. According to this evidence, the presence of two knds of variants in swine and equine viruses, which show difference in growth behavior at 42 °C, are of interest in considering the background in which new viruses may appear in man after genetic reassortment between human and animal viruses. The results obtained in the present study further increase the possibility that pigs, or other mammals such as horses, may serve as a potential reservoir for the appearance of pandemic virus in man acting as a bridging host between birds and man. Our recent study on two H1N2 recombinant viruses isolated from pigs indicates the possible occurence of recombination in swine infected dually with human and swine viruses [12, 13]. In any case genome analysis of cloned viruses suggests that the differences in growth characteristics of swine and equine viruses at high temperature may not be due to a great change of genome structure, which is consistent with previous reports that substitution of a few amino acids leads to conversion of different functions shown by the virus [7, 19, 20]. In addition, although an influenza A virus which caused a severe outbreak of acute hemorrhagic pneumonia of seals on Cape Cod Penninsula was antigenically and genetically related to avian virus [29], the evidence indicating biological relatedness of this virus to human isolates suggests the existence of potential "bridging" mammals between avian species and humans [4, 10]. It would appear that a full understanding of host specificity may be an important step in the appreciation of the ecology and emergence of human and animal influenza viruses.

References

1. Almond JW (1977) A single gene determines the host range of influenza virus. Nature 270: 617–618
2. DeWachter R, Fiers W (1972) Preparative two-dimensional polyacrylamide gel electrophoresis of ^{32}P-labeled RNA. Anal Biochem 49: 184–197
3. Giesendorf B, Bosch FX, Wahn K, Rott R (1984) Temperature sensitivity in maturation of mammalian influenza A viruses. Virus Res 1: 655–667
4. Hinshaw VS, Webster RG, Easterday BC, Bean Jr WJ (1981) Replication of avian influenza A viruses in mammals. Infect Immun 34: 354–361

5. Hinshaw VS, Webster RG, Naeve CW, Murphy BR (1983) Altered tissue tropism of human-avian reassortant influenza viruses. Virology 128: 260–263
6. Hinshaw VS, Webster RG, Rodriguez RJ (1981) Influenza A viruses: combinations of hemagglutinin and neuraminidase subtypes isolated from animals and other sources. Arch Virol 67: 191–201
7. Kawaoka Y, Naeve CW, Webster RG (1984) Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology 139: 303–316
8. Laver WG, Webster RG (1973) Studies on the origin of pandemic influenza. III. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. Virology 51: 383–391
9. Lvov DK, Zhdanov VM (1983) Persistence of genes of epidemiological influenza viruses in natural populations in USSR. Med Biol 61: 83–91
10. Murphy BR, Harper J, Sly DL, London WT, Miller NT, Webster RG (1983) Evaluation of the A / seal / Mass / 1 / 80 virus in squirrel monkeys. Infect Immun 42: 424–426
11. Murphy BR, Hinshaw VS, Sly DL, London WT, Hosier NT, Wood FT, Webster RG, Chanock RM (1982) Virulence of avian influenza A viruses for squirrel monkeys. Infect Immun 37: 1119–1126
12. Nakajima S, Sugiura A (1980) Neurovirulence of influenza virus in mice. II. Mechanism of virulence as studied in a neuroblastoma cell line. Virology 101: 450–457
13. Nerome K, Sakamoto S, Yano N, Yamamoto T, Kobayashi S, Webster RG, Oya A (1983) Antigenic characteristics and genome composition of a naturally occurring recombinant influenza virus isolated from a pig in Japan. J Gen Virol 64: 2611–2620
14. Nerome K, Yoshioka Y, Sakamoto S, Yasuhara H, Oya A (1985) Characterization of a 1980-swine recombinant influenza virus possessing H1 hemagglutinin and N2 neuraminidase similar to that of the earliest Hong Kong (H3N2) virus. Arch Virol 86: 197–211
15. Nerome K, Yoshioka Y, Torres CA, Oya A, Bachmann P, Ottis K, Webster RG (1984) Persistence of Q strain of H2N2 influenza virus in avian species: antigenic, biological and genetic analysis of avian and human H2N2 viruses. Arch Virol 81: 239–250
16. Ogawa T, Ueda M (1981) Genes involved in the virulence of an avian influenza virus. Virology 113: 304–313
17. Palese P, Schulman JL (1976) Differences in RNA patterns of influenza A viruses. J Virol 17: 876–884
18. Penseart M, Ottis K, Vandeputte J, Kaplan MM, Bachmann PA (1981) 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
19. Robertson JS, Naeve CW, Webster RG, Bootman JS, Newman R, Schild GC (1985) Alterations in the hemagglutinin associated with adaptation of influenza B virus to growth in eggs. Virology 143: 166–174
20. Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC (1983) Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature 304: 76–78
21. Scholtissek C, Bürger H, Kinstner O, Shortridge KF (1985) The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology 147: 287–294
22. Scholtissek C, Rohde W, Von Hoyningen V, Rott R (1978) On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87: 13–20
23. Scholtissek C, Rott R, Orlich M, Harms E, Rohde W (1977) Correlation of pathogenicity and gene constellation of an influenza A virus (fowl plaque) I. Exchange of a single gene. Virology 81: 74–80
24. Schulman JL, Palese P (1977) Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells. J Virol 24: 170–176

25. Shortridge KF, Butterfield WK, Webster RG, Campbell CH (1977) Isolation and characterization of influenza A viruses from avian species in Hong Kong. Bull WHO 55: 15–20
26. Stuart-Harris CH, Schild GC (1976) Influenza viruses of lower animals and birds. In: Stuart-Harris CH, Schild GC (eds) Influenza, the viruses and the disease. Edward Arnold, London, pp 78–95
27. Tobita K, Sugiura A, Enomoto C, Furuyama M (1975) Plaque assay and primary isolation of influenza A virus in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med Microbiol Immunol 162: 9–14
28. Ueda M, Tobita K, Sugiura A, Enomoto C (1978) Identification of hemagglutinin and neuraminidase genes of influenza B virus. J Virol 25: 685–686
29. Webster RG, Hinshaw VS, Bean WJ, VanWyke KL, Geraci JR, St Aubin DJ, Petursson G (1981) Charcterization of an influenza A virus from seals. Virology 113: 712–724
30. Webster RG, Morita M, Pridgen C, Tumova B (1976) Ortho- and paramyxoviruses from migrating feral ducks: Characterization of a new group of influenza A viruses. J Gen Virol 32: 217–225
31. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG (1978) Intestinal influenza: Replication and characterization of influenza viruses in ducks. Virology 84: 268–278
32. WHO Memorandum (1980) A revision of the system of nomenclature for influenza viruses. Bull WHO 58: 585–591

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