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Comparison of influenza viruses isolated from man and from whales

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Four isolates of influenza virus strains from Moscow and Habarovsk that caused outbreaks of influenza in November and December 1977 in several cities of the USSR were studied and their haemagglutinins and neuraminidases were compared with those of other human and animal influenza viruses including A/whale/Pacific Ocean/76. In HI tests these isolates, designated A/USSR/77, reacted with immune serum against A/FM/1/47 (H1N1) to the homologous titre, and with antiserum against A/whale/PO/19/76 virus to 1/8 of the homologous titre. In neuraminidase inhibition tests all A/USSR/77 isolates showed the presence of human N1 type neuraminidase, more closely related to A/sw/New Jersey/76 (Hsw1N1) than to A/FM/1/47 (H1N1) virus. The haemagglutinin of A/whale/Pacific Ocean/19/76 virus occupies an intermediate position between HO and H1, but its neuraminidase is close to Nav2. The virus from whales multiplies better at low (28°C) and at high (40°C) temperatures than do the viruses of human origin that were tested.

An epidemic caused by a virus characterized antigenically as H1N1 was first detected in the far east region of the USSR in early November 1977. The initial outbreak and the subsequent nationwide epidemic has been described previously (*1*). During 1975–76 lung and liver specimens were collected from the striped whale (Balaenopteridae) in the South Pacific and influenza A viruses were isolated from them that were shown also to have H antigens related to the H0, H1 group. In this paper we describe the antigenic and some biological properties of current H1N1 viruses isolated from man and compare them with those of the viruses isolated from the whales.

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MATERIALS AND METHODS

Viruses

Strains A/Moscow/0778/77, A/Moscow/0782/77, and A/Moscow/0897/77 were isolated on 21–24 November 1977, from young adult patients in Moscow by A. M. Ošerovič. Strain A/Habarovsk/03466/77 was isolated on 24 November 1977, from a sick child in Habarovsk by V. I. Reznik. These strains are designated A/USSR/77. After isolation in chick embryos, the strains were passaged only twice.

Specimens from striped whales (Balaenopteridae) were collected in 1975–76 by scientists with a whaler flotilla in the South Pacific. Seventy-two specimens from lungs and 65 from livers were taken from 72 whales. The specimens were stored at –20°C for 4 months in 50% glycerol prepared in phosphate buffer (pH 7.2) with antibiotics (10 000 IU of penicillin and 1500 IU of streptomycin per ml). After inoculation into chick embryos in this laboratory, 13 strains of influenza virus were isolated from the lung specimens and 1 strain from a liver specimen. The viruses were isolated at second passages.

Table 1. Reference sera used

Antiserum		Source of sera	Obtained from
Antigenic formula	Virus		
HON1	A/PR/8/34	goat	R. Webster
	A/Bel/42	goat	R. Webster
HONav1	A/WSN-A/duck/Engl/56	rat	Institute of Virology
HOH1Nav2	A/whale/PO/19/76	rabbit	Institute of Virology
H1N1	A/FM/1/47	goat	R. Webster
	A/Moscow/0897/77	rabbit	Institute of Virology
Hsw1N1	A/Sw/Iowa/13/30	rat	Institute of Virology
	A/Sw/Wisc/67	goat	R. Webster
	A/NJ/76	rat	Institute of Virology
Hsw1N1	A/NJ/76-A/PR/34 (x-53)	rabbit	E. Kilbourne
H2N2	A/Sing/1/57	goat	R. Webster
H3N2	A/HK/1/68	goat	R. Webster
	A/P.Ch/1/73	rat	Institute of Virology
H3N1	A/HK/1/68-A/PR/34 (R-2069)	rabbit	E. Kilbourne
Heq1Neq1	A/eq/Prague/56	rat	Institute of Virology
Heq2Neq2	A/eq/Miami/63	rat	Institute of Virology
Hav1N1	A/chick FPV/Rostock/34	goat	R. Webster
Hav2Nav2	A/turk/England/63	rat	Institute of Virology
Hav3Nav1	A/duck/England/56	rat	Institute of Virology
Hav4Nav1	A/duck/Czechoslovakia/56	rat	Institute of Virology
Hav5Nav2	A/tern/SA/61	rat	Institute of Virology
Hav6Neq2	A/turkey/Canada/63	rat	Institute of Virology
Hav6Nav5	A/shearwater/Australia/71	rat	Institute of Virology
Hav7Neq2	A/duck/Ukraine/63	rat	Institute of Virology
Hav7N1	A/tern/Turkmenia/73-A/Bel/42 (R-6a)	rabbit	Institute of Virology
Hav8Nav4	A/turkey/Ontario/67	rat	Institute of Virology
Hav9Nav2	A/turkey/Wisc/66-A/tern/SA/61	rabbit	E. Kilbourne

Immune sera

Immune sera to the strain A/whale/Pacific Ocean/19/76 was prepared by immunization of white rats. All 14 isolates were neutralized with this serum diluted 1:2560 in HI tests and 1:30 in NI tests, so were considered identical. Therefore, only the prototype strain A/whale/Pacific Ocean/19/76 was used in further studies. The reference sera used are listed in Table 1.

Serological reactions

Haemagglutination inhibition (HI) tests were performed by a method described previously (*2*). The sera were first treated with standard preparations of receptor-destroying enzyme. Neuraminidase inhibition (NI) tests were carried out by the method described by Webster & Pereira (*3*).

Electron microscopy

Purified and concentrated virus preparations were used. The preparations were layered and contrasted with a 1% uranyl acetate solution in methanol.

Biological properties

The methods used, such as cultivation at various temperatures, elution characteristics, plaque-forming capacity, etc., have been described previously (*4*).

All the strains studied in polyacrylamide gel were cultivated in chick embryos. Virus was concentrated by differential centrifugation and purified in linear 15–60% sucrose gradients and centrifuged at 2400 *g* for 16 h at +5°C. The purified virus was stored at –70°C.

Electrophoresis in polyacrylamide gel

Polypeptides were analysed in sodium dodecylsulfate (SDS) containing 10% polyacrylamide gel, using a tris–glycerol system. The samples were treated for 2 min at 100°C in lysis buffer containg 0.1 mol/litre dithiothreitol, 2% SDS, 10% glycerol, and 0.2% bromophenol blue. Samples containing 100 μg of virus protein in 20 ml were layered on gel and electrophoresis was performed at 40 V for 17–18 h. After electrophoresis, the gel was stained with 0.2% amido black and washed with a mixture of ethanol, acetic acid, and water (5:1:5 respectively).

Polyacrylamide gel analysis of virus-specific proteins synthesized in the infected cells

Dog kidney (MDCK) cells in monolayer culture were infected at the rate of 50 $TCID_{50}$/cell. After labelling, the cells were washed with buffered saline (0.15 mol/litre NaCl, 0.01 mol/litre tris-hydrochloride, pH 7.5), dissolved in lysis buffer heated for 2 min at 100°C, layered on top of the gradient (6–12%) polyacrylamide gel slab, and incubated under Eagle's minimal essential medium (MEM) for 5 h at 37°C. Then the culture fluid was discarded and the cells were washed three times with Hanks' balanced salt solution (BSS) and incubated further in the presence of ^{14}C-*Chlorella* hydrolysate (740 kBq/ml in Hanks' BSS) for 90 min.

RESULTS

The object of the first experiments was to determine the antigenic properties of the influenza viruses isolated from whales, of which the prototype was strain A/whale/PO/19/76.

The results of the H1 tests are shown in Table 2. It can be seen that the haemagglutinin of A/whale/PO/19/76 virus cross-reacts with immune sera to the antigenic complex H0-H1-Hsw1-Hav5 and that it is more closely related to H0 of the strain A/Bel/42 (1/16 of the homologous titre) than to H0 of the earlier strain A/PR8/34 (1/32) or to H1 of A/FM/1/47 (1/32). Thus, the haemagglutinin of this virus may be said to occupy an intermediate position between H0 and H1. Antiserum to virus A/whale/PO/19/76 reacts with virus A/USSR/77 to 1/8 of the homologous titre (Table 5).

Table 2. HI tests with A/whale/Pacific Ocean/19/76 virus [a]

Immune sera to haemagglutinins		Reciprocal titres of immune sera with: homologous virus	A/whale/PO/19/76
H0	A/PR8/34	640	20
H0	A/Bel/42	1280	80
H1	A/FM/1/47	640	20
Hsw1	A/sw/Iowa/30	1280	10
Hav5	A/tern/SA/61	80	< 10
A/whale/PO/19/76 [b]		2560	2560
A/Moscow/0782/77 [c]		2560	< 10

[a] Negative results were obtained with immune sera to haemagglutinins H2, H3, Heq1, Heq2, Hav1, Hav2, Hav3, Hav4, Hav5, Hav6, Hav7, Hav8, Hav9.
[b] Titres with antigen A/USSR/77: 320
A/PR8/34: 320
A/FM/1/47: 160
A/Bel/42: 320
A/WSN/33: 320
[c] Titres with antigens A/PR/8/34, A/Bel/47, A/WSN/33: < 10
A/FM/1/47: 640

Table 3. NI tests with A/whale/Pacific Ocean/19/76 virus [a]

Immune sera to neuraminidase		Reciprocal titres of immune sera with: homologous virus	A/whale/PO/19/76
Nav2	A/Tern/SA/61	30	30
Nav2	A/tern/Turkm/18/73	30	20
Nav2	R-136 (Hav9Nav2)	60	30
A/whale/PO/19/76		30	30

[a] Negative results were obtained with immune sera to neuraminidases N1, N2, Neq1, Neq2, Nav1, Nav3, Nav4, Nav5, Nav6.

Table 3 presents results of neuraminidase inhibition tests with the whale virus. It can be seen that the neuraminidase of A/whale/PO/19/76 is closely related or identical to neuraminidase Nav2. Thus A/whale/PO/19/76 possesses an antigenic formula (H0-H1 Nav2), not known before for human or animal influenza viruses.

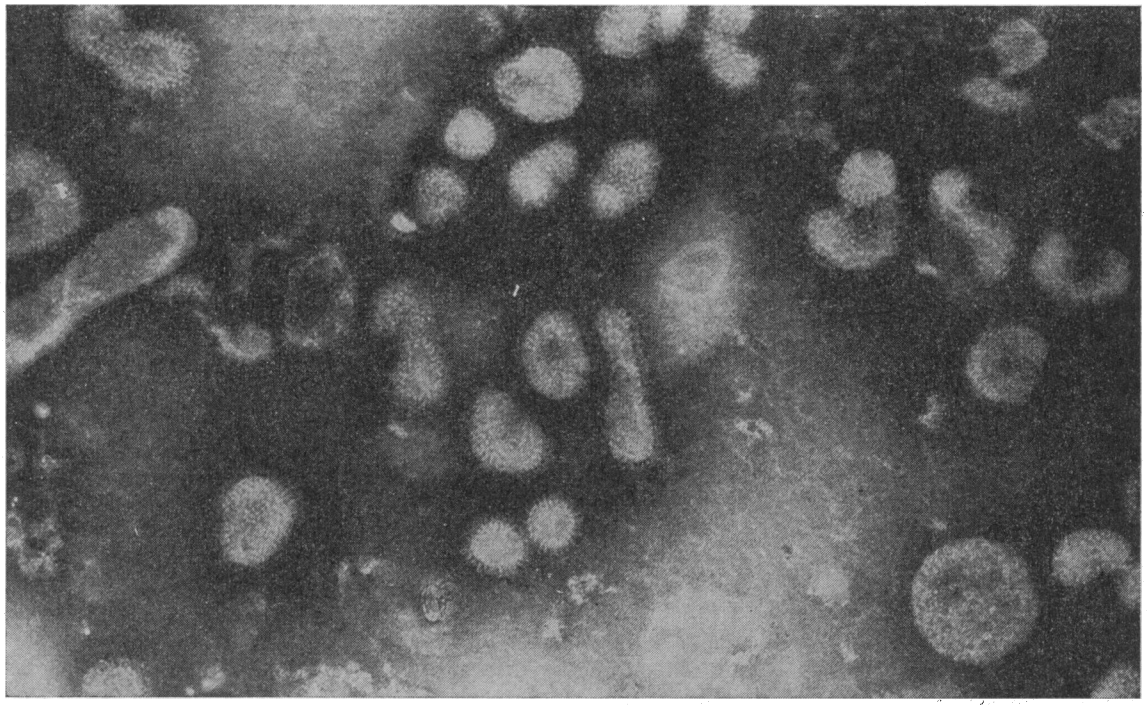

Fig. 1. Electron micrograph of virions of A/whale/Pacific Ocean/19/76 virus. Negative contrasting.

Electron-micrographs (Fig. 1) show that the morphology of the virus does not differ from that of other influenza A viruses. Both round and thread-like or irregular virions are observed. Typical structures of ribonucleoprotein are also observed in some preparations as well as spikes of haemagglutinin.

Some of the biological properties of A/whale/PO/19/76 virus and A/WSN/33 (H0N1) and A/FM/1/47 (H1N1) viruses were also compared. Table 4 shows: (*a*) the whale virus multiplies better at low (28°C) and at high (40°C) temperatures than do the two other viruses, (*b*) it is not sensitive to inhibitors in normal horse serum, (*c*) it produces turbid plaques in chick embryo fibroblast monolayers, (*d*) its haemagglutinin is moderately thermostable, and (*e*) its elution speed is intermediate. The heat resistance of the haemagglutinin of the whale virus is intermediate between that of A/WSN/33 and that of A/FM/1/47 and A/USSR/77. Virus A/USSR/77 is as sensitive to inhibitors in normal horse serum as is A/FM/1/47, but it does not form plaques on chick embryo fibroblast cultures.

The results of the study of haemagglutinins of viruses isolated during the outbreaks in November 1977 are shown in Table 5. It can be seen that the haemagglutinin of the newly isolated viruses is closely related or identical to H1 of the prototype virus A/FM/1/47 and partly (1/8) to the haemagglutinin of the whale virus. Negative results were obtained with other haemagglutinins of human and animal influenza viruses (except for some crossing with A/Bal/42).

Table 6 shows the results of the study of the neuraminidase of the recently isolated influenza viruses. It can be seen that their neuraminidase is more related to that of A/sw/New Jersey/76 (Hsw1N1) virus than to that of A/FM/1/47 (H1N1) virus.

In the polyacrylamide gel pattern of virus-specific proteins synthesized in MDCK cells, the most striking difference between A/FM/1/47 and both Port Chalmers and the newly isolated strain A/Habarovsk/77 was a much greater mobility of NS protein (Fig. 2). Any differences in mobility of the HA were barely detectable. No differences could be revealed in the mobilities of NP and M proteins.

Table 4. Some biological properties [a] of A/whale/Pacific Ocean/19/76 virus as compared with some influenza A viruses of human origin

Virus	Titre of haemagglutinin	Sensitivity to inhibitors in horse serum	Plaques	rct (log_{10} ID_{50})			T_{56} of haemagglutinin (min)	E_{rc} (hours)
				28°C	37°C	40°C		
A/whale/PO/19/76 (H0-H1Nav2)	640	—	+ turbid	8.0	8.5	8.0	60	2
A/WSN/33 (H0N1)	640	—	+ clear	5.2	7.4	6.5	10	4
A/FM1/47 (H1N1)	320	+	+ clear	6.5	7.5	6.0	120	1
A/USSR/77 (H1N1)	320	+	—	5.0	7.2	not tested	80	not tested

[a] rct = reproduction in chick embryos at various temperatures; T_{56} = thermoresistance of haemagglutinin at 56°C; E_{rc} = 100 % elution from chicken erythrocytes.

Neither neuraminidase (NA) nor P-proteins could be identified in the gels against the background of cellular proteins.

Thus, the newly isolated epidemic strains have the antigenic formula H1N1. The haemagglutinin therefore corresponds to that of the early H1 variant, although there are some crosses with the late H0 variant and H0–H1 of the whale virus, while the neuraminidase is more similar to genealogical precursors of H1N1 virus.

The polypeptides of the epidemic strain A/Moscow/0897/77 and of the virus A/whale/PO/19/76 were compared with those of the human viruses A/PR/8/34, A/FM/1/47, and A/WSN/33 by electrophoresis

Table 5. Identification of haemagglutinins of newly isolated epidemic influenza viruses (A/USSR/77) [a]

Immune sera to haemagglutinins	Reciprocal titres of sera with viruses:			
	A/Moscow 0778/77	A/Moscow 0782/77	A/Moscow 0897/77	Homologous
H0 A/PR8/34	< 10	< 10	20	640
H0 R-3a (H0Nav2)	< 10	< 10	< 10	2560
H0 R-5a (H0Nav2)	< 10	< 10	< 10	1280
H0 A/Bel/42	80	40	80	1280
H0–H1 A/whale/PO/19/76	320	320	320	2560
H1 A/FM/1/47	640	640	640	640
H2 A/Sing/57	< 10	< 10	< 10	1280
H3 A/HK/68	< 10	< 10	< 10	5120
H3 A/P.Ch./73	< 10	< 10	< 10	1280
H3 A/Tokyo/75	< 10	< 10	< 10	160
H3 A/Vict./75	< 10	< 10	< 10	160

[a] Negative results were obtained with immune sera to haemagglutinins Hsw1, Heq1, Heq2, Hav1, Hav2, Hav3, Hav4, Hav5, Hav6, Hav7, Hav8, Hav9.

Table 6. Identification of neuraminidase of newly isolated epidemic influenza viruses (A/USSR/77) [a]

Immune sera to neuraminidases	Reciprocal titres of sera with viruses:				
	A/Moscow 0778/77	A/Moscow 0782/77	A/Moscow 0897/77	A/Habarovsk 034561/77	Homologous
N1 A/FM/1/47	60	30	20	30	90
N1 A/NJ/76	60	20	60	30	30
N1 x-53	20	30	60	30	120
N1 A/FPV/Rostok	20	30	60	< 10	not tested
N1 R-6a	20	20	30	< 10	30
N1 R-2069	30	20	20	20	60
N2 A/Sing/57	< 10	< 10	< 10	n.t.	60
N2 A/HK/68	< 10	< 10	< 10	n.t.	60
N2 A/P.Ch./73	< 10	< 10	< 10	n.t.	100
N2 A/Tokyo/75	< 10	< 10	< 10	n.t.	40
N2 A/Vict./75	< 10	< 10	< 10	n.t.	20

[a] Negative results were obtained with immune sera to neuraminidases Neq1, Neq2, Nav1, Nav2, Nav3, Nav4, Nav5, Nav6.

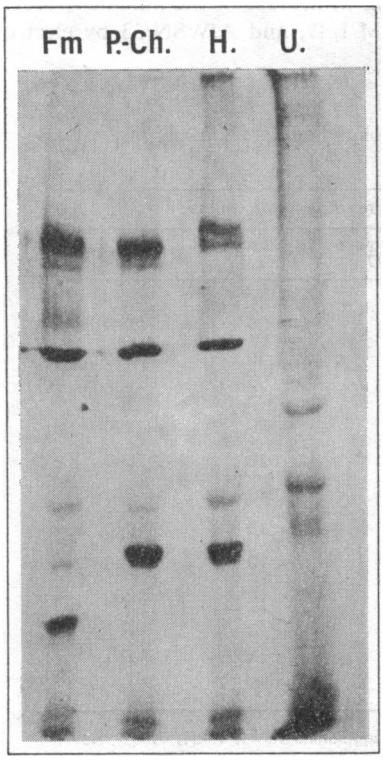

Fig. 2. Polyacrylamide gel analysis of virus-infected MDCK cells. The cells were infected at the rate of 50 $TCID_{50}$/cell and labelled with ^{14}C-*Chlorella* hydrolysate (740 kBq/ml) from 5 h to 6.5 h postinfection. Electrophoresis in gradient gel with 6–12% concentration of acrylamide for 4 h at 24 mA. U = uninfected cells; Fm = A/FM/1/47(H1N1); P.Ch. = A/Port Chalmers/1/73 (H3N2); H = A/Habarovsk/77.

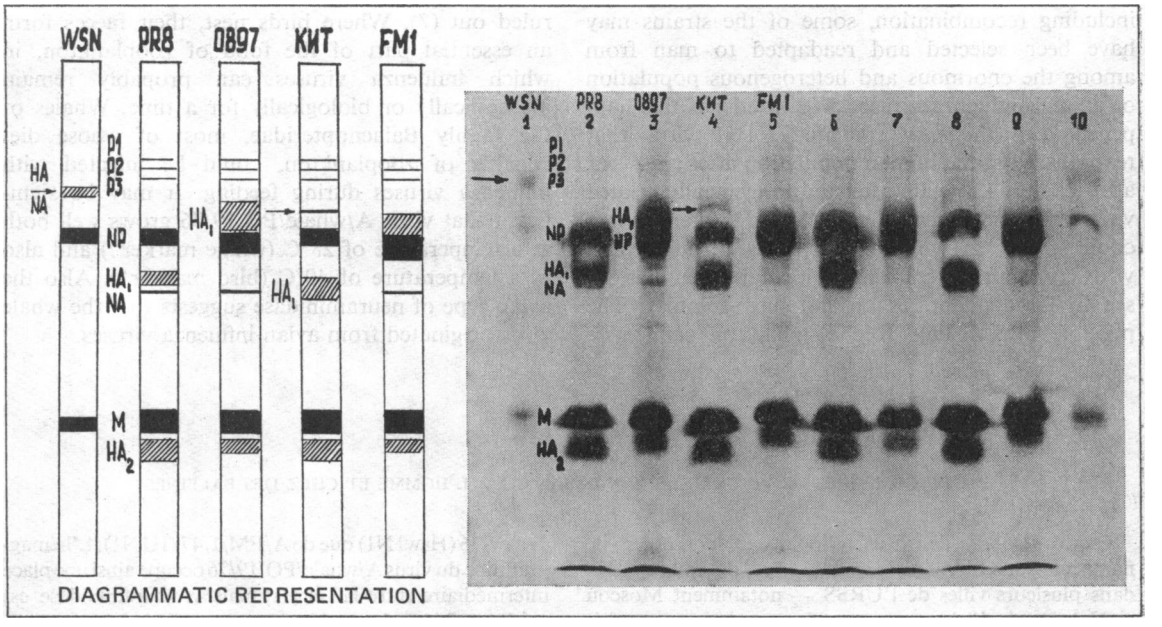

Fig. 3. Autoradiogram of electropherogram of polypeptides of human influenza viruses and A/whale/Pacific Ocean/19/76 virus. Human influenza strains: A/WSN/33—strips 1 and 10; A/PR/8/34—strips 2 and 6; A/Moscow/0897/77—strips 3 and 7; A/FM/1/47—strips 5 and 9. Influenza virus isolated from whales, A/whale/PO/19/76—strips 4 and 8. The arrows show the possible positions of neuraminidase in A/WSN/33 and A/whale/PO/19/76.

and the results are shown in Fig. 3. As can be seen, the epidemic strain A/Moscow/0987/77 has a polypeptide composition similar to that of A/FM/1/47. The composition of virus A/whale/PO/19/76, except for one polypeptide which is evidently neuraminidase, is similar to that of A/PR/8/34 virus. The polypeptide of A/whale/PO/19/76 that apparently represents neuraminidase possesses lower mobility in gel, that is, higher molecular weight, than the corresponding polypeptide of the human virus A/PR/8/34 and is evidently similar to the neuraminidase of A/WSN/33 virus. As shown in Fig. 2, both subunits of haemagglutinin (HA_2 and HA_1) of A/Moscow/0987/77 and A/FM/1/47 strains have lower mobility in the gel, that is, higher molecular weight, in comparison with the polypeptides of A/PR/8/34 and A/whale/PO/19/76 viruses.

DISCUSSION

From wild animals in the Pacific basin we isolated in 1974–76 influenza viruses that appear to be antigenically related to known human epidemic viruses. A virus of the Hong Kong complex (H3N2) was isolated from a common murre (*5*), a virus of the Asian influenza complex (H2N2) was isolated from pintail ducks (*6*) and a virus with antigens H0–H1Nav2 was isolated from whales.

Analysis of the epidemic strain A/Moscow/0897/77 in polyacrylamide gel showed that its polypeptide composition is close to that of strain A/FM/1/47 (H1N1). The virus A/whale /PO/19/76 differs from these viruses in its polypeptide composition. However, its polypeptides are similar to those of human influenza virus A/PR/8/34 (H0N1) except for one peptide, which represents neuraminidase and whose electrophoretic mobility is close to that of the neuraminidase of A/WSN/33 (H0N1) virus. The analysis of virus-specific peptides induced in MDCK cells showed that the mobility of the NS protein of A/FM/1/47 was greater than that of a newly isolated strain Habarovsk/77 and of Port Chalmers/1/73. These data are only preliminary.

Thus, it has been demonstrated that in natural biocoenoses of the region where new epidemic human influenza viruses have often arisen, old viruses that disappeared 10–20 years ago still exist. It is probable that as a result of complex ecological processes,

including recombination, some of the strains may have been selected and readapted to man from among the enormous and heterogenous population of animal influenza viruses. We wonder if this happened with the new epidemic H1N1 virus that reappreared in the human population after a 20-year absence and 1.5 years after an antigenically related virus had been isolated from whales. Influenza virus could spread to whales from birds through their virus-containing excrements, which are shed into the sea in large quantities around bird colonies. The possible intermediate role of plankton cannot be ruled out (7). Where birds nest, their faeces form an essential part of the food of zooplankton, in which influenza viruses can propably remain mechanically or biologically for a time. Whales of the family Balaenopteridae, most of whose diet consists of zooplankton, could be infected with influenza viruses during feeding. It may be significant that virus A/whale/PO/19/76 grows well both at a temperature of 28°C (whale marker?) and also at a temperature of 40°C (bird marker?). Also the avian type of neuraminidase suggests that the whale virus originated from avian influenza viruses.

RÉSUMÉ

ÉTUDE COMPARÉE DE VIRUS GRIPPAUX ISOLÉS CHEZ L'HOMME ET CHEZ DES BALEINES

Quatre isolats de virus grippaux responsables de flambées survenues en novembre et décembre 1977 dans plusieurs villes de l'URSS — notamment Moscou et Habarovsk d'où proviennent les souches — ont fait l'objet de travaux visant à comparer leurs hémagglutinines et leurs neuraminidases avec celles d'autres virus de la grippe humaine et animale — A/whale/Pacific Ocean/19/76 en particulier. Dans les épreuves d'inhibition de l'hémagglutination, ces isolats — A/USSR/77 — ont été neutralisés par un immunsérum contre A/FM/1/47 (H1N1) de titre homologue et ont réagi de façon partielle avec l'antisérum contre A/whale/PO/19/76 (1/8 du titre homologue). Dans les épreuves portant sur les neuraminidases en revanche, les neuraminidases de type humain N1 mises en évidence dans tous les isolats A/USSR/77 se sont révélées plus proches de A/sw/New Jersey/76 (Hsw1N1) que de A/FM/1/47 (H1N1). L'hémagglutinine du virus A/whale/PO/19/76 occupe ainsi une place intermédiaire entre H0 et H1, mais sa neuraminidase est voisine de Nav2. La souche provenant des baleines se multiplie mieux à faible (28°C) et à haute (40°C) températures que les virus d'origine humaine cultivés dans les mêmes conditions.

Dans les biocénoses naturelles de la région où sont apparus les nouveaux virus à l'origine d'épidémies de grippe humaine, on a pu établir la persistence d'anciens virus qui ne s'étaient pas manifestés depuis 10-20 ans. Ainsi, il est possible qu'à la suite de processus écologiques complexes et notamment de recombinaison, la sélection de certaines souches et leur réadaptation à l'homme se soient opérées au sein de l'énorme population hétérogène des virus de grippe animale.

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