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Human Trials with Wild-Type H1N1 and Recombinant H3N2-H1N1 Influenza A Viruses of 1977-1978

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A series of trials was conducted in which wild-type A/USSR/90/77 (H1N1) influenza A virus and a few of its antigenic variants were inoculated into volunteers. Infections readily occurred in people of all ages who had initial low antibody titers, but clinical effects were generally mild in comparison with those of the previously tested subtypes, H0N1, H1N1, H2N2, and H3N2. There was, however, an inverse relationship between severity of symptoms and age of volunteers, although the incidence of virus excretion and of increase in anti-hemagglutinin was apparently not age related. Naturally occurring recombinant viruses with H3 hemagglutinin and one or more genes of A/USSR/90/77-like strains were likewise studied in volunteers. These clones also produced mild symptoms, providing evidence of an attenuating effect on H3N2 viruses by the substitution of some of its genes with the genes of an H1N1 virus.

Epidemics of influenza A subtype H1N1 occurred in the interior of China in May 1977 and spread to Russia and Hong Kong a few months later (18, 29, 33). The reemergence of an H1N1 pandemic virus only 21 years after a related subtype had been displaced by the Asian (H2N2) virus was wholly unexpected. Anti-H1 and anti-N1 antibodies were widespread in people over the age of 25 years and theoretically might have been expected to place a barrier against the dissemination of an H1N1 agent. In subsequent laboratory studies, the virus was in fact shown to bear an especially close antigenic and genetic relationship to the Scandinavian H1N1 strains of 1950 and 1951 (16, 21, 28). Nevertheless, the virus was transmitted rapidly throughout the world, although overt clinical illness remained largely confined to people less than 25 years old. Sometimes H1N1 outbreaks took place concurrently with outbreaks of H3N2 viruses of the A/Victoria/3/75 and A/Texas/1/77 serotypes (14, 15, 30, 32). Antigenic drift was detected in the first year of the new H1N1 virus era, and one variant (A/Brazil/11/78) then became the dominant strain in North and South America (7). Because of the many curious features of the recent pandemics, we administered to volunteers a number of A/USSR/90/77-like strains and their antigenic variants and also several recombinants derived from mixed natural infections with H3N2 and H1N1 viruses. In this paper, we examine the human infectivity and virulence of these viruses. By comparing infectivity and virulence in these and wild-type strains from earlier subtypes, we attempted to obtain a long-term perspective of epidemic human influenza.

MATERIALS AND METHODS

H1N1 viruses wild type. A/USSR/92/77 and A/Hong Kong/119/77 viruses were both supplied to the Common Cold Unit by G. C. Schild; these viruses had histories of four egg passages only, including final passages at the terminal dilution in specific pathogen-free eggs (Table 1). A/Hong Kong/123/77, which was isolated at the Center for Disease Control, Atlanta, Ga., in specific pathogen-free eggs from a throat swab provided by W. K. Chang, Queen Mary Hospital, Hong Kong, was cloned by two successive terminal-dilution passages in specific pathogen-free eggs, followed by an additional passage at a moderate multiplicity of infection. This virus received one more passage in specific pathogen-free eggs at the Common Cold Unit before being given to volunteers (Table 1). The antigenic variants A/Brazil/11/78 and A/Lackland AFB/3/78 have been described elsewhere (14); Kendal et al. (14) also described A/Arizona/14/78, of which A/Bangkok/28/78 was the antigenic equivalent. These three viruses (Table 2) received small numbers of egg passages and were not cloned. The clones of A/Wyoming/1/78 (H1N1) and A/Lakenheath/387/78(H1N1), which were isolated at the same time as the corresponding recombinant viruses shown in Table 3 but were genotyped as A/USSR/90/77(H1N1) viruses, were grouped with the earlier H1N1 viruses shown in Table 1. Each virus had a small number of egg passages (15; see below). A/Wyoming/2/78 (Table 1), a A/USSR/90/77-like wild-type strain, was passed four times in eggs.

Viruses: recombinants. The isolation of a mixture of influenza A(H3N2) and influenza A(H1N1) from a schoolboy in Wyoming and the techniques for

TABLE 1. *Responses of volunteers to inoculations with wild-type H1N1 influenza A viruses (serotype A/USSR/90/77)*[a]

Virus	No. of egg passages	No. of volunteers	Avg age (yr)	Virus dose (log EID_{50})[b]	No. of virus excretors	No. with the following clinical reactions:[c]					Avg score of clinical reactions[d]	No. of HI increases[e]
						Severe	Moderate	Mild	Very mild	Nil		
A/USSR/92/77	4	4	40.2	7.0	2	0	1	0	3	0	8.0	2/4
A/Hong Kong/119/77	4	5	18.6	7.2	5	0	2	0	0	3	11.6	4/5
A/Hong Kong/123/77	5	3	29.3	8.5	2	0	2	1	0	0	34.3	2/3
A/Wyoming/1/78 clone 3	XE2[f]	6	49	6.2	4	0	0	1	1	4	3.3	3/6
A/Wyoming/2/78	4	6	36	7.0	3	0	2	1	1	2	15.5	5/6
A/Lakenheath/387/78 clone 1	6	5	21.8	7.7	1	1	2	1	1	0	26.2	2/5

[a] In Tables 1, 2, 3, and 7 volunteers were adults (ages, 18 to 50 years) initially free of HI antibody (5).
[b] EID_{50}, 50% egg infective dose.
[c] The clinical reactions were graded as follows: severe, typical or modified influenza; moderate, systemic and local symptoms; mild, local symptoms only; very mild, trivial effects.
[d] The average score for all clinical reactions was 13.0.
[e] Number with antibody increases/number tested.
[f] XE2, Small number of unrecorded egg passages and two recorded egg passages.

TABLE 2. *Responses of volunteers to inoculations with H1N1 viruses isolated in 1978, which showed significant antigenic changes from A/USSR/90/77*[a]

Virus	No. of egg passages	No. of volunteers	Avg age (yr)	Virus dose (log EID_{50})[b]	No. of virus excretors	No. with the following clinical reactions:[c]					Avg score of clinical reactions[d]	No. of HI increases[e]
						Severe	Moderate	Mild	Very mild	Nil		
A/Brazil/11/78	7	9	41	8.3	4	0	1	3	3	2	9.9	7/9
A/Bangkok/28/78	XE2[f]	5	31	8.5	1	0	3	0	2	0	17.8	3/4
A/Lackland AFB/3/78	XE4	1	20	8.7	1	0	1	0	0	0		0/1

[a] See reference 14.
[b] EID_{50}, 50% egg infective dose.
[c] See Table 1, footnote *c*.
[d] The average score for A/Brazil/11/78 and A/Bangkok/28/78 was 12.7.
[e] Number with antibody increases/number tested.
[f] XE2, Small number of unrecorded egg passages and two recorded egg passages.

cloning viruses of the antigenic compositions H3N2, H3N1, and H1N1 have been described previously (12, 15). The viruses A/Lakenheath/387/78 and A/Miyagi/7/78 (Table 3) were isolated from people under 26 years of age who had had influenza-like illnesses during mixed outbreaks of A/Texas/1/77 (H3N2)-like and A/USSR/90/77 (H1N1)-like infections in England and Japan, respectively (32; unpublished data). Each virus showed unusual properties during initial attempts at identification by hemagglutination inhibition (HI), which suggested the possible presence of a mixture of H3 and H1 particles. Cloning procedures were similar to those used for the original mixed isolates from Wyoming (12, 15). Clone 1 (H1N1) of the Lakenheath strain was obtained by passing a 1:10 dilution of the harvest of a second egg passage of the mixed isolate in the presence of A/Texas/1/77 (H3N2) antisera (anti-HA + anti-NA) and then repeating the maneuver. A final passage was made with a $10^{-8.0}$ virus inoculum in the presence of the same antisera. Clone 2 (H3N2) was obtained similarly in the presence of A/USSR/77 (H1N1) anti-HA and anti-NA antisera. Clone 4 (H3N1) was detected in the fourth egg passage of the isolate, which reacted predominantly as this serotype. It was finally cloned by two additional egg passages ($10^{-5.0}$ and $10^{-7.0}$ dilutions) in the presence of serum containing A/USSR/72/77 anti-hemagglutinin and A/Texas/1/77 anti-neuraminidase antibodies. A final passage was made at a $10^{-8.0}$ dilution without antiserum to obtain a seed acceptable for volunteer inoculations. A/Miyagi/7/78 (32) clone 3 (H3N1) was derived from the second egg passage of the original virus isolate by performing two successive terminal-dilution passages without antiserum. An additional two passages were made at a $10^{-4.0}$ dilution to obtain a seed for use in volunteers.

H0N1 and H1N1 influenza A viruses, 1943 to 1953. These viruses (Tables 4 and 5) were kindly provided by J. J. Skehel, National Institute for Medical Research, London, England. They were very old material, but had recorded passage histories. A/USA/43 (nine egg passages) was propagated twice in volunteers and once more in eggs before being administered to volunteers. A/Fort Warren/1/50 initially

TABLE 3. *Responses of volunteers to inoculations with wild-type recombinant H3 viruses cloned from isolates recovered during mixed H3N2-H1N1 epidemics in the United States, at a U.S. Air Force base in England, and in Japan*[a]

Virus	No. of egg passages	No. of volunteers	Avg age (yr)	Virus dose (log EID_{50})[b]	No. of virus excretors	No. with the following clinical reactions:[c]					Avg score of clinical reactions[d]	No. of HI increases[e]
						Severe	Moderate	Mild	Very mild	Nil		
A/Wyoming/1/78												
Clone 1 (H3N1)	5	7	25.8	6.8	5	0	2	4	1	0	16.7	7/7
Clone 2 (H3N2)	XE2[f]	12	32.7	6.5–8.5	7	1	2	0	2	7	7.9	8/12
A/Lakenheath/387/78												
Clone 2 (H3N2)	6	6	21.8	8.5	1	0	0	0	2	4	2.2	4/6
Clone 4 (H3N1)	8	4	39.2	8.0	0	0	0	0	0	4	0.5	2/4
A/Miyagi/7/78 clone 3 (H3N1)	XE2	5	32.4	8.5	2	0	1	0	1	3	8.3	4/5

[a] See references 12, 15, and 32.
[b] EID_{50}, 50% egg infective dose.
[c] See Table 1, footnote *c*.
[d] The average score for all clinical reactions was 7.9.
[e] Number with antibody increases/number tested.
[f] XE2, Small number of unrecorded egg passages and two recorded egg passages.

TABLE 4. *Responses of volunteers to inoculations with early H0N1 and H1N1 viruses of 1943 to 1953*[a]

Virus	No. of egg passages	Virus dose (log EID_{50})[b]	Volunteers			No. with the following clinical reactions:[c]					Avg score of clinical reactions[d]	No. of HI increases[e]
			No.	Avg age (yr)	No. of virus excretors	Severe	Moderate	Mild	Very mild	Nil		
A/USA/43	10[f]	6.0	4	20.5	3	2	1	0	0	1	32.0	3/4
A/Fort Warren/1/50(S)	>20	7.0	6	22.5	6	0	2	2	0	2	14.9	1/5
A/Madrid/3/51(L)	7	6.8	6	19	6	5	0	0	0	1	45.8	4/4
A/Eire/1/51(S)	5	7.0	4	38	2	1	1	2	0	0	33.7	4/4
A/England/1/53	6	7.8	5	19	4	5	0	0	0	0	37.4	4/5
A/England/2/53	7	8.0	4	29	1	0	1	2	1	0	17.2	1/4
A/Kentucky/302/53	6	8.0	4	44	0	0	0	0	2	2	5.2	2/4
A/Nederland/5/53	6	6.5	3	18	3	0	1	1	0	1	16.3	3/3

[a] The recorded passage histories may not always be accurate. L and S in 1950 and 1951 viruses denote the Liverpool and Scandinavian serotypes, respectively, according to the classification of Isaacs et al. (11).
[b] EID_{50}, 50% egg infective dose.
[c] See Table 1, footnote *c*.
[d] The average score for all clinical reactions was 27.6.
[e] Number with antibody increases/number tested.
[f] This virus received two human passages in addition.

came from the United States and had about 25 egg passages. The other viruses were said to have had five to seven egg passages (Table 4).

Analysis of virion RNAs. Because the migration patterns of virion ribonucleic acids (RNAs) on polyacrylamide gels vary between closely related strains (sometimes between isolates obtained from a single outbreak), these alone could not positively identify the parental origins of the genes of the recombinant viruses in the absence of pure parent viruses. Hence, RNA hybridization was used to confirm the identities of the genes in at least one virus from each mixture so as to calibrate the electropherograms of the virion RNAs.

Isotope labeling and slab gel electrophoresis of virion RNAs under different conditions and hybridization of viruses and complementary RNAs have been described elsewhere (13, 25, 26).

Analysis of temperature sensitivity. Viruses were titrated by plaque formation in MDCK cells in the presence of trypsin at 34 and 39°C (14; Table 6). The temperature of the 39°C incubator did not fluctuate by more than ±0.2°C, as monitored by a certified thermometer in the neighborhood of the flasks.

Volunteer trials. Volunteer trials were carried out by established methods (3, 5). Briefly, clinical reactions were graded severe (influenza-like and pyrexia), moderate (local and constitutional symptoms), mild (local symptoms only), very mild (trivial symptoms), and nil. A clinical scoring system based on coryza, pyrexia, handkerchief count, and subjective symptoms was useful for making direct comparisons between complete trials, but in general was found to be less reliable than the grading and subject to distortion by single high scores when applied to small volunteer groups (six volunteers or fewer). Viruses were given in high dosages, ranging from $10^{6.2}$ to $10^{8.7}$ 50% egg infective doses per volunteer to ensure maximum responses and to overcome partial protection which was not reflected in pretrial HI titers. Experience had shown

TABLE 5. *Summarized data on virulence for volunteers at the Common Cold Unit of some wild-type human influenza A viruses ranging chronologically from 1943 to 1978*

Virus	Surface antigens	Maximum clinical effect in HI antibody-free volunteers	References
A/USA/43	H0N1	Influenza	This paper (Table 6)
Seven strains isolated from 1950 to 1953	H1N1	Influenza, but some variation between viruses	This paper (Table 6)
A/England/501/68	H2N2	Influenza	2
A/Hong Kong/1/68, A/England/878/69, A/England/939/69, A/Port Chalmers/1/73, A/Scotland/840/74	H3N2	Influenza	1, 4, 6
A/New Jersey/8/76	Hsw1N1	Mild upper respiratory effects, not apparently age related	3; Beare et al., in press; unpublished data
Pandemic viruses of 1977	H1N1	Mild upper respiratory effects inversely related to age	This paper (Tables 1, 2, and 7)

TABLE 6. *Temperature sensitivity of influenza A H3N2 and H1N1 isolates and of clones obtained from a mixed H3N2-H1N1 isolate*

Virus	No. of tests	EOP (%)[a]
Wild-type virus		
A/Victoria/3/75 (H3N2)	4	120–500
A/Hong Kong/117/77 (H1N1)	2	0.01
A/USSR/90/77 (H1N1)	2	1.5–15
A/Hong Kong/123/77 (H1N1)	2	0.03–003
Recombinant virus		
A/Wyoming/1/78 clone 1 (H3N1)	2	12–25
A/Wyoming/1/78 clone 2 (H3N2)	2	13–16

[a] Efficiency of plaque formation (EOP) is defined as plaque-forming units per milliliter at 39°C/plaque-forming units per milliliter at 34°C in MDCK cells with 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone–trypsin per ml of agar overlay.

that reproducible results could be obtained by balancing volunteer groups according to their initial HI titers alone (5); the reciprocals of these titers were in this case 24 or less against the homologous H1N1 strain or against A/Texas/1/77(H3N2), as appropriate (Tables 1 through 3 and 7). Very few volunteers had titers of more than 12. Viruses were administered intranasally in 1.0-ml amounts by the customary dropping method, and in most trials nasal washings were collected for virus isolation on days 3 and 4 after challenge. Each washing was then inoculated allantoically into four 11-day-old embryonated hen eggs, which were incubated at 33°C for 2 days and then tested for HA with 0.5% fowl erythrocytes.

Serology. HI and neuraminidase-inhibiting antibody titrations on sera of volunteers were performed by standard techniques. Neuraminidase inhibition tests were used only in initial screening for antibodies as a guide to their incidence and were found to be generally (although not entirely) absent. Neuraminidase-inhibiting antibody was not found in the absence of anti-H1 antibody, but anti-N2 antibody was sometimes present. HI antibody titers were assessed simultaneously on paired sera (treated with cholera filtrate) which were collected before the trials and 2 or 3 weeks afterwards.

RESULTS

Trials with wild-type A/USSR/90/77 (H1N1)-like viruses. The effects of inoculating three 1977 and three 1978 H1N1 viruses into 29 volunteers at the Common Cold Unit are shown in Table 1. The 1978 strains had been recovered during simultaneous H3N2 and H1N1 outbreaks in Wyoming and at a U.S. Air Force base in Lakenheath, England (Table 3), but were antigenically and genetically indistinguishable from prototype A/USSR/90/77 (unpublished data). Although the number of volunteers in each of the groups was smaller than we desired, in the virtual absence of detectable HI and neuraminidase-inhibiting antibodies maximum infectivities and clinical and serological responses were undoubtedly achieved. A single influenza-like illness (severe reaction) was recorded, about 60% of the people shed virus, and a similar proportion developed HI increases. The reactions were significantly milder than the reactions with wild-type viruses of subtypes H3N2, H2N2, H1N1, and H0N1 (4, 8, 26) (Tables 4 and 5), all of which would be expected to produce something approaching febrile influenza in the majority of seronegative patients.

Epidemic and nonepidemic variants of A/USSR/90/77-like virus. Antigenic analyses of several hundred virus isolates in the United States toward the end of the influenza season in 1978 revealed the existence of a number of minor antigenic variants (14). Therefore, the new virus was clearly established as an epidemic subtype, and immunological drift may have been hastened by the widespread incidence of HI antibodies in older people. One variant (reference strain A/Brazil/11/78) was also detected in South America, where it became dominant. Because A/Brazil/78 displaced A/USSR/77 in South America within 1 year of the reap-

TABLE 7. *Combined responses analyzed according to the ages of the 44 volunteers in Tables 1 and 2 who received the wild-type H1N1 viruses*

Ages of volunteers (yr)	No. inoculated	No. of virus excretors	No. with the following clinical reactions:[a]					Avg score of clinical reactions[b]	No. of HI increases
			Severe	Moderate	Mild	Very mild	Nil		
18–24	16	11	1	9	2	1	3	25.4	8/15(53)[c]
24–30	4	2	0	2	0	1	1	17.2	3/4
30–40	4	3	0	2	1	1	0	19.0	4/4
40–50	20	7	0	1	4	8	7	6.7	13/20(65)

[a] See Table 1, footnote *c*.
[b] The average score of for all reactions was 15.6.
[c] Number with antibody increases/number tested. The numbers in parentheses are percentages.

pearance of H1N1 influenza, this virus too was inoculated into antibody-free volunteers (Table 2); a second variant, A/Bangkok/28/78, was similarly studied in a human trial. (This virus resembled the prototype A/Arizona/14/78 [14], which had been isolated in monkey kidney tissue culture and was therefore unsuitable for human use.) The virulence of these viruses for humans was low and was not significantly different from that of the A/USSR/90/77-like viruses. Randomization of volunteers for inoculation with yet another variant, A/Lackland AFB/3/78, unfortunately resulted in the inclusion of only one antibody-free individual in the trial (Table 2).

Wild-type recombinant viruses containing the HA of contemporary H3N2 viruses and some other genes of A/USSR/90/77-like virus. Several instances were reported of mixed infections with H3N2 and H1N1 influenza viruses during circulation of both subtypes (15, 32). A number of clones, which were characterized immunologically as H3N2 or H3N1, were recovered from these virus mixtures, and each of these also contained one or more non-H non-N genes of H1N1 viruses (see below). These clones were inoculated into volunteers free of anti-H3 antibody (Table 3) and, like the H1N1 viruses (Tables 1 and 2), induced relatively mild clinical effects, although they remained infective and antigenic. These responses were quite different from those which had been obtained previously with wild-type H3 viruses (1, 6) (Table 5). Very high doses of virus were administered (up to $10^{8.5}$ 50% egg infective doses) to ensure that the natural and acquired resistances not reflected in the H1 screening were fully overcome, but it was clear that the five strains tested were basically attenuated. A/Lakenheath/387/78 clone 2 containing H3N2 antigens was in fact less virulent than clone 1 (H1N1) recovered from the same virus mixture (Table 1).

Genotyping of recombinant viruses. Because of the low pathogenicity of the H3 viruses (Table 3), their gene analyses were of special interest. Comparisons of migration patterns of RNA segments and hybridizations of RNAs showed that all of the viruses, with the exception of A/Lakenheath/387/78 clone 2, behaved like cloned recombinants containing nucleoprotein from an H3N2 parent and matrix and nonstructural proteins from an H1N1 parent (Table 9). In addition, the polymerase genes (RNAs 1, 2, and 3) of the Wyoming clone 1 (H3N1) and clone 3 (H3N2) and Lakenheath clone 4 (H3N1) viruses were of mixed origin. The origins of the HA and neuraminidase genes of all of the viruses (previously identified by serological methods) were confirmed by migration on gels. On the basis of gel electrophoresis with several clones from the same original mixture, A/Miyagi/7/78 clone 3 (H3N1) appeared to derive its nucleoprotein from an H3N2 parent and its RNAs 2 and 3, neuraminidase, and matrix and nonstructural proteins from an H1N1 parent; one large RNA (RNA 1) was provisionally assigned to the H3N2 parent. An examination of the electropherograms of A/Lakenheath/387/78 clone 2, however, revealed the presence of four bands in the region of RNAs 1 through 3 and two bands in the region of RNA 8, which suggested that cloning was incomplete and that the virus remained a mixture containing two copies of some genes. This was confirmed by hybridization experiments in which cells infected with this virus produced complementary RNA that hybridized almost completely with viral RNA 8 of both A/USSR/77 and A/Texas/77 (Table 8). Hybridizations also confirmed the existence of nonstructural protein gene (RNA 8) of H1N1 origin in the other recombinant clones.

Effect of age on clinical responses of infection with wild-type H1N1 viruses. Repeated reports that recent H1N1 influenza epidemics produced their maximum impact on children and young adults (14, 18, 29, 30) were supported by an analysis according to age of the trials at the Common Cold Unit with H1N1 viruses (Table 7). Our volunteers ranged in age

TABLE 8. *RNA hybridization experiments which were used to confirm the derivation of gene 8 in recombinant viruses*[a]

Complementary RNA of:	% Hybridization with ^{32}P virion RNA 8 of:	
	A/Texas/1/77	A/USSR/90/77
A/Texas/1/77(H3N2)	100	56
A/USSR/90/77(H1N1)	67	100
A/Wyoming/1/78 clone 1 (H3N1)	ND[b]	105
A/Lakenheath/387/78 clone 2 (H3N2)[c]	90	94
A/Lakenheath/387/78 clone 4 (H3N1)	74	105
A/Miyagi/7/78 clone 3 (H3N1)	64	106

[a] ^{32}P-labeled RNA was obtained after acrylamide gel electrophoresis of virion RNA and was hybridized at 80°C in the presence of 1% formaldehyde with complementary RNA extracted from infected chicken embryo fibroblasts (13, 25, 26).

[b] ND, Not done.

[c] Acrylamide gel electrophoresis of viron RNA from this virus indicated the presence of two bands in the region of RNA 8, suggesting that cloning was incomplete.

from 18 to 50 years, and there was a sharp difference between the severity of clinical symptoms in the 18- to 23- and 40- to 50-year age groups. Of 16 younger people, 10 (62%) sustained severe or moderate reactions, but such reactions were observed in only 1 of 20 older people (5%). Mean clinical scores were 25.4 and 6.7, respectively, although the incidences of HI increases (53 and 65%) were not obviously different. We found virus excretions in 11 of 16 (69%) and 7 of 20 (35%) individuals, respectively.

Temperature sensitivity of wild-type H1N1 viruses. It was predicted by Mackenzie (19) that conditional lethal mutant viruses with temperature-sensitive (*ts*) defects would invariably be less virulent than the *ts*$^+$ strains from which they were derived, irrespective of the genes in which their mutations occurred. This view was independently stated by Mills and Chanock (20), who attributed this phenomenon to a rising temperature gradient within the respiratory tract, extending from the nose to the lungs and leading to a shutoff of virus growth at the lower levels. Recombinants inheriting *ts* genes from a mutagenized virus and HA from contemporary wild-type strains would therefore prove relatively attenuated and might function as live vaccines. In general, this has been correct in practice (22). It was reported that a high proportion of the new H1N1 viruses were naturally occurring *ts* strains (18; C. M. Chu, personal communication). Therefore, we performed a limited study on a possible connection between temperature sensitivity and the relatively low human virulence of some of the the H1 and H3 viruses that we had tested (Table 6). The control, the wild-type virus A/Victoria/3/75 (H3N2), was not *ts*, whereas A/Hong Kong/123/77 (H1N1) was unequivocally so. A/Hong Kong/117/77 was moderately *ts*, whereas the growth of A/USSR/90/77 was not seriously reduced at 39°C. This was also true of A/Wyoming/1/78 clone 1 (H3N1) and clone 2 (H3N2). Therefore, there was no obvious relationship between the *ts* phenotype and the lack of virulence of these viruses. However, a clone of A/Hong Kong/123/77 (H1N1) selected at 39°C in bovine kidney tissue culture by B. R. Murphy was virulent in volunteers at the Common Cold Unit (Beare, unpublished data). This is being investigated. It seemed unlikely that there could be a direct connection between virulence and shutoff temperature of wild-type human influenza viruses, but clearly more data were required.

Early wild-type H1N1 viruses in volunteers. Because recent H1N1 viruses seemed to be unusual among human influenza subtypes, it was important to compare them as closely as possible with other wild-type viruses and in particular with their counterparts of 1946 to 1956, of which they were evidently near relatives (16, 21, 26, 28). A number of virulent H1N1 viruses of 1951 to 1953 had already been characterized in volunteers as part of a study in 1977 to identify old virulent viruses from which to develop experimental live vaccines (Table 4). The data are not comparable in quality to those in Tables 1 and 2, but it was possible to conclude that in general this subtype was more virulent than the subtype which appeared in 1977, although there was again a suggestion of more severe clinical symptoms in young patients. There was also considerable variation in virulence generally. The strain A/Fort Warren/1/50, an S-type virus (11), was included because of its close similarity to A/USSR/90/77 (16, 21, 28), although its passage history was less than ideal for our purposes.

In Table 5 we summarize the data accumulated for volunteers at the Common Cold Unit concerning the virulence for humans of influenza A viruses ranging chronologically from 1943 to 1978. Inevitably, these data are fragmentary. A/USA/43(H0N1), which was said to have had nine egg passes, was passed twice in volunteers, after which it induced very unpleasant influenza-like symptoms. The more recent subtypes, H2N2 and H3N2, produced unequivocal clinical influenza (1, 2, 4, 6), whereas A/New Jersey/8/76 (Hsw1N1), although of low virulence, never became established as a human pathogen. There-

fore, it seemed that A/USSR/90/77 and its variants were alone among wild-type pandemic viruses in their reduced virulence for adult humans and in their apparent lack of virulence for humans of middle age.

DISCUSSION

The reestablishment of pandemic H1N1 influenza followed by the rapid appearance of antigenic drift has added yet another enigma to the many curiosities of influenza history. In 1977, the time was not ripe for the recycling of this virus (9); furthermore, widespread prevalence of protective antibodies placed such an event outside the range of prediction. Perhaps only a limited number of antigenic subtypes can cause epidemics, but there is no obvious explanation for the explosive spread of H1 in 1977, whereas in the previous year, Hsw remained confined to a single small outbreak in New Jersey. The virulences of the two viruses are similar (A. S. Beare, A. P. Kendal, and J. W. Craig, J. Med. Virol., in press), but only elderly people tend to have residual anti-Hsw antibodies, whereas in 1977 anti-H1 antibodies were common in everyone except children and young adults.

It may be that the overriding reason for the recent contrasting epidemiology of the two subtypes is the age-related difference in clinical responses to infection with the new H1N1 viruses (Table 7). Transmission of influenza viruses is probably not governed solely (or even mainly) by the quantity of virus shed since previous volunteer studies showed that the New Jersey HswlN1 virus was freely excreted for periods of up to 8 days (3; Beare et. al., in press). However, reactions were always mild, whereas reactions induced by H1N1 infection (Table 7) were more marked in young patients. This observation is consistent with the fact that children have borne the main brunt of A/USSR/90/77-like epidemics whereas older people have suffered little morbidity. The relatively low virulence of H1N1 viruses for large sections of the population may also be responsible for failure of these viruses to totally supplant the H3N2 subtype, of which there have been sporadic isolations well into 1979 (31).

Immunological priming of older people has undoubtedly contributed to the less severe clinical effects that they suffer during H1N1 infections (Table 7). However, a virus-specific cause may also be present, since in our trials the early H1N1 virus A/Eire/1/51 (similar to Scandinavian viruses, such as A/Fort Warren/1/50) did induce typical influenza in middle-aged people (Table 4). Therefore, old and new H1N1 viruses may be biologically different despite the evidence from recent laboratory work that there are very close biochemical and antigenic relationships between A/USSR/90/77 and the viruses of the winter of 1950 and 1951 (16, 21, 26, 28). Other evidence for virus-specific factors in virulence included the finding that several early H1N1 viruses cause more severe illness in young persons than recent H1N1 strains. The precise connection between virulence and transmissibility can only be conjectured. Spread could simply be promoted by the coughing and sneezing which is a consequence of infection with a virulent virus, but it is more likely that there is an undefined genetic link between the two properties. If this is so, the parallels between human and animal influenzas may not always be close, since viruses are frequently disseminated among pigs with minimal clinical effects (17) and epizootics of avian influenza are not necessarily associated with high pathogenicity (10). In addition, our studies showed that the A/Brazil/11/78-like variant which replaced A/USSR/90/77-like strains in South America is not noticeably more infectious or virulent than its predecessor (Table 2); therefore, we have no explanation for its rapid spread in that area in April to May 1978, rather than a variant like A/Lackland AFB/3/78, which shows evidence of a greater degree of antigenic drift away from A/USSR/77 (14, 28).

In recent years, much attention has been focused on recombination as the means by which pandemic viruses are generated in nature (27). However, there is no evidence for the operation of this mechanism in the initial appearance of A/USSR/77-like viruses (21, 26). When recombination did occur between these viruses and the prevalent H3N2 strains (15, 32), the effect was evidently a frequent reduction in the virulence of the H3 viruses, even when infectivity was retained (Table 3). The attenuation of the recombinants which we tested can be ascribed neither to host range nor to *ts* mutations in the genes of one of the parents (6, 22); although the circumstances under which these viruses were isolated did not permit the recovery of true parental H3N2 virus for characterization, it is very exceptional for wild-type H3N2 viruses not to cause influenza symptoms in some volunteers, and one can probably assume that the H3 parents of these recombinants were in fact virulent.

Our findings with three different sets of recombinants containing the H3 HA suggest that, as with avian viruses (23), simple reassortment of genes of human wild-type viruses may lead to formation of gene constellations that do not permit the expression of virulence. Alternatively, reassortments of virulent genes in wild-type H3N2 viruses and avirulent genes in wild-type H1N1 viruses can have the same effect (6),

TABLE 9. *Derivation of genes in recombinant viruses recovered from mixtures of wild-type influenza A (H3N2) and A (H1N1) strains*

Gene	Derivation in recombinant virus:				
	A/Wyoming/1/78		A/Lakenheath/387/78[b]		A/Miyagi/7/78 clone 3 (H3N1)[c]
	Clone 1 (H3N1)[a]	Clone 3 (H3N2)	Clone 4 (H3N1)	Clone 2 (H3N2)	
RNA 1	H1N1	H3N2	H1N1	H3N2	—
RNA 2	H3N2	H3N2	H3N2	H3N2	H1N1
RNA 3	H1N1	H1N1	H1N1	M	H1N1
HA	H3N2	H3N2	H3N2	H3N2	H3N2
Neuraminidase	H1N1	H3N2	H1N1	H3N2	H1N1
Nucleoprotein	H3N2	H3N2	H3N2	H3N2	H3N2
Matrix protein	H1N1	H1N1	H1N1	H1N1	H1N1
Nonstructural protein	H1N1	H1N1	H1N1	M	H1N1

[a] H1N1, Derived from influenza A (H1N1) virus; H3N2, derived from influenza A (H3N2) virus; M, possible mixture of genes from influenza A (H1N1) and A (H3N2) viruses; —, results could not be interpreted. Results were obtained initially by acrylamide gel electrophoresis of virion RNAs under several conditions, in which different recombinant clones from the same mixed infection were compared with each other and with wild-type H3N2 and H1N1 viruses. Confirmation was obtained by RNA-RNA hybridization. Functional assignments for RNAs 1, 2, and 3 have not been made for this, and the other, viruses.

[b] Determined by electrophoresis of virion RNAs through a urea-containing gel at 30°C.

[c] Determined by electrophoresis of virion RNAs through a urea-containing gel at 27°C.

particularly when the genes concerned with RNA synthesis are of mixed parentage (24) (Table 9). However, recent observations that viruses from epidemics in North American in 1978 and 1979 contained an HA from an A/Brazil/11/78-like virus and that all of the genes coding for the proteins involved in RNA synthesis were from an H3N2 parent (P. Palese, personal communication; W. J. Bean and N. J. Cox, unpublished data) do demonstrate that recombinants inheriting a mixed complement of genes from current wild-type H3N2 and H1N1 viruses are not necessarily without epidemic potential. Further studies on the virulence of recombinants prepared from human wild-type parents have been initiated because of the relevance of the experiments to influenza epidemiology and because of their manifest importance for the development of live vaccines.

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