INFECTION AND IMMUNITY, May 1981, p. 693-697
0019-9567/81/050693-05$02.00/0

Vol. 32, No. 2

Evaluation of A/Alaska/6/77 (H3N2) Cold-Adapted Recombinant Viruses Derived from A/Ann Arbor/6/60 Cold-Adapted Donor Virus in Adult Seronegative Volunteers

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The influenza A/Ann Arbor/6/60 (H2N2) cold-adapted (*ca*) virus was evaluated as a donor of attenuating genes to new variants of influenza A virus. This *ca* donor virus was mated with the A/Alaska/6/77 (H3N2) wild-type virus, and three A/Alaska/6/77 (H3N2) *ca* recombinant viruses were produced. The parental origin of the genes in the three *ca* recombinants had been determined previously (2), and their virulence for adult seronegative volunteers was assessed in the present study to identify the genes present in the *ca* donor virus that confer attenuation. Each of the recombinants received the hemagglutinin and neuraminidase genes from the A/Alaska/6/77 (H3N2) wild-type parent. One *ca* recombinant (CR-29) received all six transferable genes from the *ca* parent and was found to be satisfactorily attenuated in the volunteers. The two other *ca* recombinants received five of the six transferable genes with a wild-type gene at the *M* or *NS* locus. The pattern of infection in humans with these latter two *ca* recombinants was similar to the CR-29 *ca* recombinant. These findings demonstrate that inheritance of a gene in *ca* recombinants at the *M* or *NS* locus segregates independently of attenuation and suggest that the *M* and *NS* genes present in the *ca* donor virus are not the major determinants of attenuation conferred by this virus.

The influenza A/Ann Arbor/6/60 cold-adapted (*ca*) virus is being evaluated as a donor of attenuating genes to new variants of influenza A virus (1, 7-10, 14). This *ca* virus replicates efficiently at 25°C, a temperature restrictive for growth of wild-type virus, and is temperature sensitive (*ts*) (7, 20, 21). Several *ca* recombinant viruses which were derived from the A/Ann Arbor/6/60 *ca* virus were attenuated for animals and humans (3, 4, 9, 10, 13, 15). These *ca* recombinants had received the hemagglutinin and neuraminidase surface glycoprotein genes from the wild-type virus and five or six of the remaining transferable genes from the A/Ann Arbor/6/60 donor virus (2, 5).

The A/Ann Arbor/6/60 gene(s) that confer attenuation have not as yet been identified. It is important to determine which A/Ann Arbor/6/60 gene(s) confers attenuation and to identify these attenuating genes in future *ca* recombinant viruses derived from this parent. To determine the contribution that each A/Ann Arbor/6/60 gene makes to attenuation, one would ideally like to transfer one of the six nonsurface antigen genes to a wild type and assess the effect this gene has on the virulence of the virus for humans. Since all *ca* recombinant viruses produced to date have received the majority of the six transferable genes from the *ca* parent, an alternative method of analysis must be carried out. This method requires that a *ca* recombinant of a new variant bearing all six transferable A/Ann Arbor/6/60 genes be produced and found to be attenuated for susceptible adults.

Subsequently, a *ca* recombinant virus derived from the same parents is identified that has only five of the six transferable genes derived from the *ca* parent, i.e., one of the six genes from the *ca* parent has been substituted with a gene from the wild-type virus. If the A/Ann Arbor/6/60 *ca* mutant gene at the substituted locus was a major determinant of attenuation, then the loss of this

attenuating gene should result in increased virulence of the *ca* recombinant. In the present study, such an analysis has been carried out for three A/Alaska/6/60 *ca* recombinant viruses possessing different genotypes, one virus with all six transferable A/Ann Arbor/6/60 genes and the other two viruses with a wild-type gene at the *M* (membrane protein) or *NS* (nonstructural protein) locus. The findings suggest that the A/Ann Arbor/6/60 *M* and *NS* genes do not play a major role in attenuation of recombinants derived from this *ca* donor virus.

MATERIALS AND METHODS

Viruses. The method for the production of the *ca* recombinant viruses from the A/Ann Arbor/6/60 (H2N2) *ca* and A/Alaska/6/77 (H3N2) wild-type cloned viruses has been described (2). Although the *ca* recombinants are given two different designations, CR-29 or CR-31 (Table 1), the three *ca* recombinants were isolated from three separate, but similar, recombination mixtures and, therefore, properties of the three recombinants can be compared (2). The phenotypic and genotypic characterization of the three *ca* recombinants and their parents are summarized in Table 1. The techniques for the antigenic characterization and for the determination of the *ca* and *ts* phenotypes have been described (2, 7, 20). The determination of the *ca* and *ts* phenotypes have been described (2, 7, 20). The determination of the genotype by comparing migration rates of virion ribonucleic acid from parental and recombinant viruses has been done by Cox et al. as described previously (2).

The preparation of the cloned wild-type A/Alaska/6/77 virus administered to volunteers and used as a parent for production of *ca* recombinants was described previously (12, 17). The preparation and safety testing of the A/Alaska/6/77 *ca* recombinant viruses was done as described previously (6, 11, 13).

Clinical studies. Volunteers selected for this study had a serum hemagglutination inhibiting antibody titer of 1:8 or less. The facilities at the University of Maryland School of Medicine and the University of Rochester School of Medicine that were used for isolation of the volunteers and the methods that were used for the characterization of the nasal wash specimens for virus and the determination of serum hemagglutination inhibiting, neuraminidase inhibiting, and enzyme-linked immunosorbent assay antibody responses have been described (12, 16). The enzyme-linked immunosorbent assay was used in the present study to determine if a difference in pre- and postinoculation serum antibodies developed, but not to assign a titer to the individual serum specimens as described previously (16).

RESULTS

The response of the volunteers to A/Alaska/6/77 *ca* recombinant or wild-type virus is presented in Table 2. Each of the four groups of volunteers had similar preinoculation mean serum hemagglutination inhibiting and neuraminidase inhibiting antibody titers. The wild-type virus induced illness in 50% of the volunteers and was shed for a longer duration and in higher quantity than were the *ca* recombinant viruses. The CR-29 recombinant, which received all six internal genes from the A/Ann Arbor/6/60 *ca* parent (Table 1), infected 75% of the vaccinees and induced illness in only 1 of 18 infected volunteers. This illness consisted only of coryza and myalgia without fever. The three *ca* recombinant viruses exhibited a similar level of attenuation, as indicated by a comparable reduction in duration and magnitude of the virus shedding and frequency of illness induced. The serum hemagglutination inhibiting, neuraminidase inhibiting, and enzyme-linked immunosorbent assay antibody responses were similar in each of the three groups of vaccinees, although these responses were all less than those of volunteers infected with wild-type virus.

Thirty-five isolates were recovered from the *ca* vaccinees; each retained the *ts* and *ca* phe-

TABLE 1. *Phenotypic and genotypic characteristics of A/Alaska/6/77 ca recombinant viruses*

Virus	Antigenic analysis	*ca* phenotype	Shutoff temp (°C)[a]	Parental origin of genes[b] in recombinant virus at the following RNA segment (gene product)[c]:							
				1 (ND)	2 (ND)	3 (ND)	4 (*HA*)	5 (*NA*)	6 (*NP*)	7 (*M*)	8 (*NS*)
A/Alaska/6/77 wild-type parent	$H3N2_{77}$	0	>40	WT	WT	WT	WT	WT	WT	WT	WT
A/Alaska/6/77 CR-29 clone 2	$H3N2_{77}$	+	39	AA	AA	AA	WT	WT	AA	AA	AA
A/Alaska/6/77 CR-31 clone 3	$H3N2_{77}$	+	39	AA	AA	AA	WT	WT	AA	WT	AA
A/Alaska/6/77 CR-31 clone 10	$H3N2_{77}$	+	38	AA	AA	AA	WT	WT	AA	AA	WT
A/Ann Arbor/6/60 *ca* parent	$H2N2_{60}$	+	38	AA	AA	AA	AA	AA	AA	AA	AA

[a] Shutoff temperature is defined as the lowest temperature at which a 100-fold or greater reduction of virus titer (plaque-forming units per milliliter) was observed.

[b] AA indicates gene derived from the A/Ann Arbor/6/60 *ca* parent; WT indicates genes derived from the wild-type parent.

[c] ND, Not determined; *HA*, hemagglutinin; *NA*, neuraminidase; *NP*, nucleoprotein; *M*, membrane protein; *NS*, nonstructural protein.

TABLE 2. *Response of seronegative volunteers to A/Alaska/6/77 ca recombinant or wild-type virus*[a]

A/Alaska/6/77 virus	Dose (log_{10} $TCID_{50}$)[b]	No. tested	% infected[c]	Virus shedding			Serum HAI antibody		Serum NI antibody		% with serum HAI, NI, and/or ELISA antibody response	% with indicated illness[d]:		
				% shedding	Avg duration (days ± SE)[e]	Peak (mean log_{10} titer ± SE)[f]	Pre-, postinoculation reciprocals of mean log_2 titers ± SE	% with rise in antibody ≥ fourfold	Pre-, postinoculation reciprocals of mean log_2 titers ± SE	% with 1.5 log_2 rise in antibody		Febrile (≥37.8) and/or systemic	Upper respiratory tract	Any illness
CR-29 clone 2	7.5	24	75	46	0.9 ± 0.2	1.5 ± 0.4	1.5 ± 0.2, 3.3 ± 0.4	50	1.4 ± 0.4, 2.9 ± 0.4	46	67	4[g]	4	4
CR-31 clone 3	7.7	12	100	67	1.6 ± 0.5	2.1 ± 0.5	1.3 ± 0.1, 3.1 ± 0.4	50	1.0 ± 0.4, 2.4 ± 0.5	33	83	0	0	0
CR-31 clone 10	7.7	17	94	29	0.4 ± 0.2	1.0 ± 0.1	1.4 ± 0.1, 3.5 ± 0.4	65	1.2 ± 0.5, 2.4 ± 0.6	47	82	6[g]	6	6
Wild type	4.2	8	100	100	4.9 ± 0.9	4.5 ± 0.6	1.5 ± 0.3, 3.8 ± 0.7	75	1.5 ± 0.5, 3.6 ± 0.4	75	88	38	50	50

[a] HAI, Hemagglutination inhibiting; NI, neuraminidase inhibiting; ELISA, enzyme-linked immunosorbent assay.

[b] $TCID_{50}$, 50% tissue culture infective dose.

[c] Evidence of virus shedding or an antibody response or both.

[d] Upper respiratory tract illness was defined as an illness observed by two physicians on 2 consecutive days that consisted of either or both of the following: (i) pharyngitis, the occurrence of pharyngeal erythema and discomfort; or (ii) rhinitis, the development of rhinorrhea. Systemic illness was defined as the occurrence of myalgia or chills and sweats.

[e] Data from only those volunteers infected was used for calculations. Each vaccinee was tested for 7 days, and volunteers who received wild-type virus were tested for 10 days. SE, Standard error.

[f] The amount of virus in the nasal wash specimen from each volunteer was determined and the peak titer of volunteers who shed virus was averaged.

[g] Afebrile systemic illness.

notypes, indicating that these viruses were genetically stable in adult volunteers.

Transmission of CR-31 clone 10 virus from 7 vaccinees to 2 susceptible contacts did not occur.

DISCUSSION

Cox et al. (2) have proposed that new *ca* recombinant viruses produced by mating the A/Ann Arbor/6/60 *ca* donor virus with a new epidemic influenza A virus should receive all the nonglycoprotein genes of the *ca* donor virus and the surface glycoprotein genes from the new variant. Such a gene constellation for a *ca* recombinant vaccine candidate would tend to minimize potential difficulties that might arise from unpredictable gene-gene interactions between influenza A virus genes (18, 19). *ca* recombinants with the desired gene constellation have been produced readily by mating the A/Ann Arbor/6/60 *ca* donor and wild-type viruses at 25°C, with 66% of *ca* recombinants produced in this manner receiving all six nonsurface antigen genes from their *ca* parent (2). Although it is reasonable to expect that future efforts to produce *ca* recombinants that have all six nonglycoprotein genes from the A/Ann Arbor/6/60 *ca* parent and the hemagglutinin (*HA*) and neuraminidase (*NA*) genes from the wild-type parent will also prove successful, this may not be the case. Therefore, it is essential to identify the gene(s) present in the A/Ann Arbor/6/60 *ca* parent virus that confer attenuation to insure that at least these genes are present in future *ca* recombinant viruses. Since the *RNA1*, *RNA3*, and the *NP* (nucleoprotein) genes are present in all A/Ann Arbor/6/60 recombinants that possess the *ca* and *ts* phenotypes, it is likely that one or more of these genes bear mutations that confer attenuation (2).

The data obtained in the present study suggest that the *M* and *NS* gene are not the major determinants of attenuation and are consistent with the suggestion that mutations present in the *RNA1*, *RNA2*, *RNA3*, and/or *NP* genes are the major determinants of attenuation (2). An A/Victoria/3/75 *ca* recombinant with the *RNA2*, *HA*, and *NA* genes from wild-type virus and the five other internal genes from the A/Ann Arbor/6/60 *ca* parent virus behaved clinically and virologically like the A/Alaska/6/77 CR-29 virus evaluated in the present study (13). Considered together, these results suggest that the *RNA1*, *RNA3*, or *NP* genes might be the major attenuating genes of the A/Ann Arbor/6/60 *ca* donor virus. These suggestions are offered with the reservation that it is not known whether the wild-type genes present in the *ca* recombinant viruses have undergone mutation during the recombination and subsequent cloning at 25°C. In addition, it is theoretically possible that a wild-type gene at the *M*, *NS*, or *RNA2* locus would not function efficiently in human cells with five A/Ann Arbor/6/60 *ca* genes, and this loss of efficiency of replication would result in a loss of virulence. If such a situation existed in the present or previous studies, the attenuating effect of the *M*, *NS* or *RNA2* genes of the *ca* parent would not be revealed in the clinical evaluation of the *ca* recombinants.

It had previously been suggested that the *NS* gene might be an important attenuating gene of the A/Ann Arbor/6/60 parent virus because an A/Scotland/840/70 *ca* recombinant virus with a wild-type *NS* gene retained the capacity to induce febrile illness despite having received five of the six transferable genes from the A/Ann Arbor/6/60 *ca* parent (13). This suggestion was not supported by the findings of the present study, and the explanation for this might lie in the small numbers of volunteers tested. At comparable doses (approximately $10^{7.5}$ 50% tissue culture dose per volunteer), the A/Scotland/640/74 *ca* recombinant induced febrile influenzal illness in 1 of 8 infected volunteers, whereas the A/Alaska/6/77 clone 10 recombinant induced afebrile systemic illness in 1 of 16 volunteers. Such differences are not significant. Both *ca* recombinants exhibited a pattern of viral replication in humans characteristic of other attenuated *ca* recombinant viruses, i.e., a short duration and low magnitude of virus shedding (13, 15).

An important overall requirement of this approach to producing a live influenza A virus vaccine is that new variants must be reproducibly attenuated by the acquisition of genes from the *ca* parent. *ca* recombinant viruses that were derived from the A/Queensland/6/72 (H3N2), A/Alaska/6/77 (H3N2), or A/Hong Kong/123/77 (H1N1) wild-type virus and possessed the desired gene constellation of six transferable genes from the *ca* donor virus induced only one afebrile systemic illness during infection of 66 seronegative adults (3, 13, 15). This experience is in contrast to the corresponding wild-type viruses which induced febrile or systemic illness in 8 of 14 comparable volunteers (15). In the case of the A/Hong Kong/123/77 *ca* recombinant, each of the 41 volunteers who were infected were initially doubly seronegative and, thus any residual virulence specified by the six transferable genes should have been fully expressed (15). In addition, Wright and colleagues have observed that A/Alaska/6/77 and A/Hong Kong/123/77 *ca* recombinants were satisfactorily attenuated in a limited number of doubly

seronegative children (P. Wright, personal communication). These results indicate that the six transferable genes present in the A/Ann Arbor/6/60 donor virus can render epidemic influenza A viruses belonging to two subtypes satisfactorily attenuated for fully susceptible individuals. These results form the basis for the continued evaluation of this promising influenza A donor virus.

ACKNOWLEDGMENTS

This work was supported by Public Health Service contracts NO1-AI-42553, NO1-AI-02663, and NO1-AI-80001 from the National Institute of Allergy and Infectious Diseases.

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