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Temperature-Sensitive Mutants of Influenza A Virus: Evaluation of A/Victoria/3/75-*ts*-1[E] Recombinant Viruses in Volunteers

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The Hong Kong/68-*ts*-1[E] virus and its Udorn/72 and Georgia/74 recombinants, which have a 38°C shutoff temperature and a *ts* lesion(s) on the genes coding for the P3 and NP proteins, were adequately attenuated and immunogenic in adult volunteers who lacked serum hemagglutination-inhibiting antibody (titer, ≤1:8), but who possessed serum neuraminidase-inhibiting antibody. Two Victoria/75-*ts*-1[E] clones that also had a 38°C shutoff temperature and a *ts* lesion(s) on the same two genes were administered to adult volunteers who lacked both serum hemagglutination-inhibiting antibody (titer, ≤1:8) and neuraminidase-inhibiting antibody (titer, ≤1:4). In contrast to the behavior of the earlier *ts*-1[E] recombinants, the Vic/75-*ts*-1[E] recombinants retained the capacity to cause febrile, systemic illness. However, the recombinants were attenuated compared with wild-type virus. The Vic/75-*ts*-1[E] virus vaccinees shed a larger amount of virus for a longer time than the previous *ts*-1[E] vaccinees, but they shed less virus than volunteers infected with wild-type virus. The *ts*-1[E] virus shed retained its *ts* phenotype in most instances and failed to spread to susceptible contacts. Vaccinees were partially protected against homologous wild-type virus challenge. The failure of HK/68, Udorn/72, and Georgia/74 *ts*-1[E] vaccinees to develop systemic reactions may reflect the presence of neuraminidase immunity before infection. In this situation, attenuation probably resulted from the degree of defectiveness of the *ts*-1[E] recombinant virus and the existence of neuraminidase immunity in the recipients. The 50% human infectious dose of the Vic/75 *ts*-1[E] virus was less than $10^{5.2}$ 50% tissue culture infective doses. This suggests that at the time of a pandemic shift involving both the hemagglutinin and neuraminidase glycoproteins, a small amount of live virus vaccine might be effective in initiating infection.

The influenza A Hong Kong/68-*ts*-1[E] virus (38°C shutoff temperature of plaque formation) and its Udorn/72 and Georgia/74 (38°C shutoff) recombinants have many properties that make them suitable candidate vaccine viruses. They are satisfactorily attenuated in adult volunteers who lack serum hemagglutination-inhibiting (HAI) antibody (i.e., ≤1.8), but who possess serum neuraminidase-inhibiting (NI) antibody; they are genetically stable after replication in adult volunteers; and they provide protection against homologous wild-type virus challenge (7, 8, 12, 13). However, when the HK/68 and Udorn/72 *ts*-1[E] viruses were administered to children who lacked both serum HAI and NI antibodies, febrile responses were observed, and virus which had lost the *ts* phenotype was recovered from a minority of the children (4, 15). It was not clear, however, whether the febrile reaction of the doubly seronegative child was a function of young age or of immunological inexperience with influenza A virus.

The appearance of the A/Victoria/3/75 virus allowed us to examine this question. The Vic/75 virus possesses a neuraminidase that is six- to eightfold different from the neuraminidase of the preceding A/Port Chalmers/73 virus in reciprocal tests (A. Kendal, personal observation).

Therefore, it was not surprising to find that over 90% of adult volunteers who had a serum HAI antibody titer of ≤1:8 to A/Vic/3/75 virus also had an NI antibody titer of ≤1:4. Thus, it was possible to evaluate Vic/3/75-*ts*-1[E] recombinant viruses (38°C shutoff temperature and $H3_{75}N2_{75}$ antigens) in adults who were doubly seronegative.

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MATERIALS AND METHODS

Viruses. The wild-type A/Victoria/3/75 virus was isolated in specific pathogen-free eggs as described (9). The wild-type virus suspension administered to volunteers was uncloned and had undergone two passages in specific pathogen-free eggs. The production and characterization of the A/Vic/3/75-*ts*-1[E] recombinant clones 81 and 113 were described previously (9). Briefly, they were recombinants that: (i) had a 38°C shutoff temperature of plaque formation like their HK/68-*ts*-1[E] parent; (ii) possessed the hemagglutinin and neuraminidase of the Vic/75 wild-type virus; and (iii) had the two *ts* genes of the HK/68-*ts*-1[E] virus. The Vic/75 wild-type virus from which these recombinants were derived was passaged once in eggs, seven times in primary calf kidney (BK) culture, and once more in eggs. After recombination, the Vic/75-*ts*-1[E] clones were passaged five times in BK tissue, including a plaque-to-plaque purification, before a suspension of virus for administration to volunteers was prepared in the allantoic cavity of 10-day-old SPAFAS eggs (SPAFAS, Inc., Storrs, Conn.). The suspension was shown to be free of adventitious agents as previously described (5, 7).

Viruses used in HAI tests included the A/Vic/3/75 wild-type virus and the A/Vic/3/75-A/Equi 1/Prague/56 recombinant containing the hemagglutinin of the A/Vic/75 parent and the neuraminidase of the A/Equi 1/Praque/56 virus. The reciprocal recombinant was used to measure NI antibody.

Clinical studies. The wild-type and recombinant *ts* viruses were evaluated at the Clinical Center, National Institutes of Health, Bethesda, Md.; the Clinical Research Center for Vaccine Development of the University of Maryland, Baltimore, Md.; the University of Rochester, Rochester, N. Y.; or the Baylor College of Medicine, Houston, Tex., in facilities and volunteer populations previously described (2, 3, 13). Informed consent was obtained before participation in the study. The methods used for isolation of volunteers, administration of virus, observation of clinical responses, and collection and processing of serum and nasopharyngeal washes have been described (12, 13).

Assessment of viral shedding and immune responses. The techniques for measuring infectivity and characterization of viral isolates and HAI, NI, and neutralizing antibodies have been described (1, 12–14). A Vic/75 recombinant virus containing the $H3_{75}$ hemagglutinin and the N Equi 1 neuraminidase was used in the neutralization assay. Neutralization assays and infectivity titrations were performed with rhesus monkey kidney tissue grown in Co-Star 24-well plastic tissue culture trays (Flow Laboratories, Rockville, Md.).

RESULTS

Evaluation of Vic/75 wild-type virus. The dose of Vic/75 wild-type virus to be used as a challenge virus was determined by first administering $10^{4.2}$ 50% tissue culture infective doses ($TCID_{50}$) intranasally to six seronegative (serum HAI antibody, ≤1:8) volunteers. Only 3 volunteers were infected, so an additional group of 12 seronegative individuals was inoculated with $10^{5.2}$ $TCID_{50}$ (Table 1). This dose infected each of the volunteers, and illness developed in 66%; 42% of the volunteers developed febrile and/or systemic manifestations of illness. A dose of $10^{5.2}$ was therefore chosen to challenge Vic/75-*ts*-1[E] vaccinees.

Evaluation of the *ts*-1[E] recombinants in seronegative adults. The Vic/75-*ts*-1[E] clone 81 was given to seronegative volunteers at three doses: $10^{5.2}$, $10^{6.2}$, or $10^{7.2}$ $TCID_{50}$ (Table 1). The clone 113 was tested only at $10^{6.5}$ $TCID_{50}$. At $10^{7.2}$ $TCID_{50}$, 12% of volunteers who received clone 81 developed febrile and/or systemic reactions, whereas 21% had local upper respiratory tract symptoms. Virus was shed for an average of 3.0 days with a mean $\log_{10}$ peak titer of 2.7 ($TCID_{50}$/ml of nasopharyngeal wash). Lower doses of clone 81 caused fewer symptoms, whereas the same percentage of volunteers was infected. In addition, virus was shed in smaller amounts and for a shorter period of time. With the three doses of virus administered, it was clear that clone 81 was attenuated compared with that of the Vic/75 wild-type virus, but this was especially evident at the $10^{5.2}$ dose level, at which illness was not observed with the *ts*-1[E] recombinant.

Evaluation of Vic/75-*ts*-1[E] clones 81 and 113 in ferret tracheal organ cultures revealed that clone 81 was able to replicate at 37°C more efficiently than clone 113 (9). These observations suggested that these clones might differ in reactogenicity for volunteers. Nonetheless, 1 of the 12 volunteers who received clone 113 developed a systemic reaction, and 4 had mild upper respiratory tract disease. The duration and magnitude of virus shedding by the clone 113 vaccinees did not differ significantly from that of the clone 81 vaccinees who received a comparable dose of virus ($10^{6.2}$ $TCID_{50}$). Thus, there was no significant difference between the Vic/75-*ts*-1[E] clones when evaluated in volunteers.

A total of 248 isolates from volunteers who received either clone were tested to determine if

TABLE 1. *Response of seronegative volunteers to A/Vic/3/75 temperature-sensitive or wild-type virus*[a]

Vic/75 influenza virus	Dose ($TCID_{50}$, log_{10})	No. tested	% Infected[b]	Virus shedding			Mean log_2 serum HAI antibody titer (±SE)		% With rise in titer			% Volunteers[h] with:			
				% Shedding	Avg duration[c] (day ± SE)	Peak mean log_{10} titer[d] (±SE)	Pre	Post	Serum HAI antibody[e]	Serum NI antibody[f]	Nasal wash neutralizing antibody[g]	Fever (≥37.8)	Systemic symptoms (afebrile)	Upper respiratory tract symptoms	Any illness
ts-1[E] clone 81	7.2	52	84	67	3.0 ± 0.34	2.7 ± 0.51	2.4 ± 0.10	4.3 ± 0.15	60	18	34	8	4	21	27
ts-1[E] clone 81	6.2	20	80	60	2.8 ± 0.57	2.3 ± 0.46	2.5 ± 0.13	3.8 ± 0.27	40	16	20	0	5	25	30
ts-1[E] clone 81	5.2	13	85	85	2.0 ± 0.65	1.4 ± 0.65	2.0 ± 0.19	4.3 ± 0.19	85	23	10	0	0	0	0
ts-1[E] clone 113	6.5	12	83	83	2.3 ± 0.70	1.8 ± 0.68	1.9 ± 0.23	4.4 ± 0.46	50	33	25	0	8	25	33
Wild type	5.2	12	100	100	5.0 ± 0.39	3.4 ± 0.47	2.0 ± 0.47	3.8 ± 0.56	50	45	60	25	17	67	67

[a] All volunteers had preinoculation HAI antibody titers of ≤3 (log_2). Antibody titers are reciprocals. A total of 95 to 100% of each group had NI antibody titers of ≤2 (log_2). SE, Standard error of the mean.

[b] Evidence of virus shedding and/or a rise in serum or nasal wash antibody.

[c] Each vaccinee was tested daily for 7 days; each volunteer who received wild-type virus was tested daily for 10 days.

[d] The amount of virus in the nasopharyngeal wash specimens in each volunteer was determined, and the maximum amount shed by each volunteer was averaged. The specimens from only 15 of the 52 volunteers who received $10^{7.2}$ were analyzed.

[e] Percent with ≥4-fold rise. HAI antibody titers were determined using $H3_{75}$ $N2_{75}$ virus as test antigen. Comparable fold rises were obtained using $H3_{Vic}$ N_{equiv1} virus. A rise to either antigen is presented.

[f] Percent with ≥1.5 (log_2) rise.

[g] Percent with ≥4-fold rise.

[h] Only volunteers with evidence of infection.

[i] Only 5 or 12 volunteers were tested.

the recombinants maintained their *ts* phenotype after replication in humans. Only four isolates had ts^+ virus, but ts^+ virus was not detected in the original nasopharyngeal wash of these volunteers. This finding raises the possibility that the reversion to the ts^+ phenotype may have occurred in the tissue culture used to isolate the virus and not in the volunteer. The immunological response to the two clones of virus was similar and resembled that induced by the wild-type virus.

Studies to detect transmission of vaccine virus to susceptible contacts were carried out in a dormitory setting in which the volunteers shared common facilities. Spread of wild-type virus to contacts had been observed in this setting (B. Murphy and M. Levine, unpublished data). There was not evidence of transmission of virus from 10 vaccinees who received $10^{7.2}$ $TCID_{50}$ of clone 81 virus to 4 susceptible contacts, despite the fact that 9 of the vaccinees were infected and 5 shed virus.

Response of Vic/75-*ts*-1[E] vaccinees to challenge with Vic/75 wild-type virus. Vic/75-*ts*-1[E] vaccinees who received $10^{7.2}$ $TCID_{50}$ of clone 81 were challenged 4 weeks later with $10^{5.2}$ $TCID_{50}$ of wild-type virus (Table 2). The following evidence of protection was observed: (i) fewer volunteers were infected; (ii) less virus was shed for a shorter duration; and (iii) fewer illnesses occurred. However, two vaccinees became ill, one developed a febrile illness while the other individual had an afebrile upper respiratory tract response.

Effect of neuraminidase immunity on the response of volunteers to *ts*-1[E] viruses. We had previously observed that the HK/68 *ts*-1[E] and Udorn/72-*ts*-1[E] recombinants caused a mild febrile response in serum NI antibody-negative children; this type of reaction was not observed in children who had neuraminidase immunity (4). Also, in a previous study, the presence of serum NI antibody was associated with resistance to illness caused by experimental challenge with HK/68 wild-type virus (8). For these reasons we analyzed the possible effect of neuraminidase immunity upon the response of serum HAI antibody-negative volunteers to the Vic/75-*ts*-1[E] clone 81 recombinant (Table 3). The 10 volunteers who had detectable serum NI antibody and became infected had NI antibody in low titer, i.e., 1.1 ± 0.25 (mean $\log_2 \pm$ standard error). It was not surprising, therefore, that the response of these individuals could not be distinguished from that of the serum NI-negative volunteers who became infected, except for a suggestion that virus was shed longer by the latter group.

TABLE 2. *Response of Vic/75-ts-1[E] vaccinees and seronegative controls to wild-type A/Vic/3/75 virus*[a]

Volunteer	No. tested	No. infected[b]	Virus shedding			Mean $\log_2$ serum HAI antibody titer		No. with rise in titer			No. of volunteers[h] with:			
			No.	Avg duration[c] (days ± SE)	Peak mean $\log_{10}$ titer[d] (±SE)	Pre	Post	Serum HAI antibody titer[e]	Serum NI antibody titer[f]	Nasal wash neutralizing antibody/no. tested[g]	Fever (≥37.8)	Systemic symptoms (afebrile)	Upper respiratory tract symptoms	Any illness
ts-1[E] vaccinee	9	5	3	1.8 ± 1.05	0.8 ± 0.47	3.6	4.3	2	1	3/9	1	0	1	2
Control	12	12	12	5.0 ± 0.39	3.4 ± 0.47	2.0	3.8	6	5	3/5	3	2	8	8

[a] Each volunteer received $10^{5.2}$ $TCID_{50}$ of virus intranasally. The *ts*-1[E] vaccinees were challenged 4 weeks after *ts*-1[E] virus administration.
[b] Same as Table 1 footnote *b*.
[c] Same as Table 1 footnote *c*.
[d] Same as Table 1 footnote *d*.
[e] Same as Table 1 footnote *e*.
[f] Same as Table 1 footnote *f*.
[g] Same as Table 1 footnote *g*.
[h] Same as Table 1 footnote *h*.

TABLE 3. *Effect of preinoculation serum NI antibody titer on the response of serum HAI-negative volunteers to Vic/75-ts-1[E] virus*[a]

Presence of preinoculation serum NI antibody	No. of vaccinees[b]	Avg duration of virus shedding (days ± SE)[c]	No. shedding virus for ≥5 days	No. with:			
				Fever (≥37.8)	Systemic symptoms (afebrile)	Upper respiratory tract symptoms	Any illness
No[d]	34	3.1 ± 0.4	11	4	1	7	9
Yes	10	2.6 ± 0.7	1	0	1	3	4

[a] A dose of $10^{7.2}$ $TCID_{50}$ of Vic/75-*ts*-1[E] clone 81 virus was administered. Only infected volunteers are included.

[b] Infected vaccinees only.

[c] SE, Standard error of the mean.

[d] Volunteers were considered to possess preinoculation NI antibody if their undiluted serum inhibited enzyme activity by 50%.

The possible effect of neuraminidase immunity upon response to *ts*-1[E] recombinants was examined in another manner by comparing the clinical responses of serum HAI antibody-negative volunteers (titer, ≤1:8) to the Udorn/72, Georgia/74, and Vic/75-*ts*-1[E] viruses in relation to preinoculation serum NI antibody titer (Table 4). Udorn/72 and Georgia/74 *ts*-1[E] vaccinees, who possessed NI antibody at the time of virus administration, did not develop febrile or systemic signs or symptoms. In contrast, 12% of the Vic/75-*ts*-1[E] vaccinees who lacked both HAI and NI antibodies developed fever and/or systemic manifestations of illness. This increase in reactogenicity seen in the Vic/75-*ts*-1[E] vaccinees was accompanied by a higher level of virus shedding compared with that of the two earlier *ts*-1[E] recombinants. Furthermore, there appeared to be an inverse relationship between level of prechallenge serum NI antibody and duration of virus shedding.

DISCUSSION

In previous studies the Hong Kong/68, Udorn/72, and Georgia/74 *ts*-1[E] recombinants failed to induce febrile or systemic responses in adults who were serum HAI antibody negative but NI antibody postive (7, 8, 12 13). In contrast, in the present study, the Vic/75-*ts*-1[E] recombinant viruses retained the capacity to cause a febrile response. One host factor that may have influenced this difference was preexisting neuraminidase immunity. Thus, the HK/68, Udorn/72, or Georgia/74 *ts*-1[E] vaccinees who had preinoculation serum NI antibody shed less virus and developed fewer symptoms than did the Vic/75-*ts*-1[E] vaccinees who lacked NI antibody or possessed a low titer of this antibody. In persons with some immunity to influenza A virus, primarily directed against the neuraminidase antigen, the degree of defectiveness specified by the two *ts*-1[E] lesions appeared sufficient to produce a satisfactorily attenuated, immunizing infection. In this situation, attenuation was probably achieved by the sum of two factors: the degree of defectiveness of the recombinant and the existence of neuraminidase immunity resulting from prior infection(s). In contrast, most serum HAI-negative Vic/75-*ts*-1[E] vaccinees lacked serum NI antibody. In this circumstance, the lack of neuraminidase immunity may have allowed expression of the residual virulence of the Vic/75-*ts*-1[E] recombinants.

The HK/68 and Udorn/72-*ts*-1[E] recombinants caused a transient febrile response in children who lacked serum HAI and NI antibodies (4). A similar response was seen in doubly seronegative adults given the Vic/75-*ts*-1[E] clone 81 recombinant in the present study. These observations suggest that immunological inexperience rather than age was responsible for the febrile responses seen in young children given *ts*-1[E] recombinants. Other evidence derived from evaluating an HK/68-*ts*-1[A] recombinant in children indicated that prior experience with related influenza A viruses was a major determinant of response to vaccine virus (P. F. Wright, M. Kervina, J. Thompson, A. E. Tarrence, and D. T. Karzon, Proceedings of a Symposium on International Association of Biological Standardization, Geneva, Switzerland, in press).

Factors other than NI immunity may play a role in the difference in level of attenuation of *ts*-1[E] recombinant viruses. For example, the Vic/75-*ts*-1[E] clone 81 replicated to a higher level at 37°C in tracheal organ culture than did clone 113 (9). However, in hamsters and in man significant differences between these clones were not observed, and, at a comparable dose, (about $10^{6.2}$ $TCID_{50}$) both clones induced systemic illness in volunteers. Immunological factors that were not measured (such as cell-mediated immunity) or those that were below the level of detection of our current test procedures could

TABLE 4. *Response of serum HAI antibody-negative volunteers to influenza A ts-1[E] recombinant viruses: effect of neuraminidase immunity*[a]

Virus	Dose[b] ($TCID_{50}$, log_{10})	No. tested	% Infected	Preinoculation serum antibody titer (mean log_2 ± SE)		Virus shedding			% Volunteers with ≥4-fold rise in serum HAI antibody titer	% Volunteers with:		
				HAI	NI	% Shedding	Avg duration (days ± SE)	Peak mean log_{10} titer (±SE)		Fever (≥37.8) or systemic symptoms	Upper respiratory tract symptoms	Any illness
Udorn/72-*ts*-1[E] clone 24	7.2	17	61	2.2 ± 0.18	8.7 ± 0.35	18	1.1 ± 0.67	0.5 ± 0.28	53	0	12	12
Georgia/74-*ts*-1[E] clone 2	7.0	32	97	2.0 ± 0.13	4.9 ± 0.60	66	2.3 ± 0.37	1.8 ± 0.32	60	0	16	16
Victoria/75-*ts*-[E] clone 81	7.2	52	84	2.4 ± 0.10	≤1	67	3.0 ± 0.34	2.7 ± 0.51	60	12	21	27
Victoria/75 wild type	5.2	12	100	2.0 ± 0.47	≤1	100	5.0 ± 0.39	3.4 ± 0.47	50	42	67	67

[a] Antibody titers are reciprocals. SE, Standard error of the mean.
[b] The titers of these viruses were determined all in the same test on RMK tissue and were very close to those previously determined.

have influenced the clinical response of volunteers to the Vic/75-*ts*-1[E] recombinants.

Each *ts* recombinant virus produced by mating the HK/68-*ts*-1[E] virus and a wild-type virus, such as the Vic/75-*ts*-1[E] clones 81 or 113, receives two *ts* genes (the P3 and NP genes) from the *ts*-1[E] parent (11) and the hemagglutinin and neuraminidase genes from the wild-type virus. However, at the other four known loci, genes could come from either parent. In the HK/68-*ts*-1[E] parent, these remaining four genes were derived from the HK/68 wild-type virus (11). Hence, in recombinants of HK/68-*ts*-1[E] these genes were always wild type, derived either from HK/68-*ts*-1[E] or the wild-type parent. This suggests that these genes did not play a role in attenuation of the *ts*1[E] recombinants. Nonetheless, it is possible that certain combinations of *ts* genes and wild-type genes at these four loci might affect the level of attenuation of recombinant H3N2 viruses. The genotype of the *ts*-1[E] recombinant viruses is being determined, and the role that "gene constellations" might play in attenuation of the *ts*-1[E] recombinants is being examined.

In other ways, the Vic/75-*ts*-1[E] clones behaved like the earlier *ts*-1[E] recombinants (7, 12, 13). They were predominantly genetically stable, nontransmissible, and protective against wild-type virus challenge. In previous studies the protection afforded against illness by HK/68 and Udorn/72 *ts*-1[E] virus was complete after challenge with homologous wild-type virus, whereas two illnesses were seen in Vic/75-*ts*-1[E] vaccinees (7, 13). The reasons for this difference remain undetermined.

A dose response evaluation of the Vic/75 wild-type virus and the Vic/75-*ts*-1[E] virus indicated that the 50% human infectious doses (HID_{50}) of the two viruses were comparable, i.e. HID_{50} of the wild-type virus was $10^{4.2}$ $TCID_{50}$ and that of the *ts* virus was less than $10^{5.2}$ $TCID_{50}$. The HID_{50} of the Vic/75-*ts*-1[E] virus was 10-fold lower than that of the HK/68-*ts*-1[E] virus (8). This suggests that in adults NI immunity can decrease the infectivity of a virus in addition to modulating clinical response. Consistent with this interpretation was the observation that a quantity of *ts* virus noninfectious for adults with NI antibody was infectious for children who lacked both HAI and NI antibodies (7, 15). Thus, at the time of a pandemic shift involving both the hemagglutinin and neuraminidase glycoproteins, the quantity of vaccine virus required to immunize may be significantly lower than is the case during interpandemic periods when neuraminidase immunity (and low levels of hemagglutinin immunity as well) provide resistance to infection.

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