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Aerosol Vaccination of Mice with a Live, Temperature-Sensitive Recombinant Influenza Virus

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Mice were vaccinated intranasally (i.n.) or with small-particle aerosols (SPA; 2 μm) or large-particle aerosols (LPA; 8 μm) of an attenuated, temperature-sensitive, recombinant A influenza (H3N2) virus, ts-1[E]. Serum virus-neutralizing and hemagglutination inhibition antibodies were detected for all vaccinated mice by 28 days. Bronchoalveolar wash fluids had increased levels of immunoglobulin (IgG, IgA) only in the i.n.-vaccinated mice. Hemagglutination and virus-neutralizing antibodies were detected in the SPA- and i.n.-vaccinated groups but not in the LPA vaccinates. Upon challenge with SPA of a mouse virulent H3N2 influenza virus, total protection was obtained for the SPA- and i.n.-vaccinated mice, whereas only 89% of the LPA group survived. Replication of the challenge virus was significantly repressed in both the lower and upper respiratory tracts of the three groups of vaccinated mice compared to the nonvaccinated controls. The protection afforded the SPA- and i.n.-vaccinated mice was the same as measured for mice after recovery from earlier sublethal active infection with virulent virus.

Inactivated vaccines long used for immunoprophylaxis against influenza have been variably effective (18). Recent studies have indicated that more effective immunization against influenza may be engendered by the intranasal (i.n.) instillation of live, attenuated, temperature-sensitive (ts) and recombinant viruses (1, 6, 9, 10, 17). In theory, attenuated vaccine strains proliferate in the respiratory tract to induce an immune response without overt clinical illness (9). Depending on the shut-off growth temperature of a specific ts mutant, the i.n.-administered virus replicates efficiently in the cooler upper respiratory tract (32 to 34 C), with moderate or no proliferation at the 37 C temperature of the lung (10). The ts mutants tend to replicate with lessened efficiency at temperatures approaching their shut-off temperatures (37 to 40 C) at which the parent strain grows normally. Instillation i.n. also applies the antigen directly to the mucosal surfaces of the respiratory tract for the stimulation of local antibody (20).

Previous studies with various attenuated ts recombinant influenza viruses in animals have all involved i.n. administration (9, 10). In the present work, we have investigated, for the first time, the immunogenicity of a live, attenuated, ts-1[E] recombinant influenza virus (H3N2) presented to mice in two aerosol particle sizes deposited in different ways in different regions of the pulmonary tree. There also was the need to determine whether vaccination by the i.n. or aerosol route would protect against a small-particle aerosol (SPA) challenge of virulent virus, a manner by which infections probably occur in nature. The experimental design, therefore, consisted of exposing groups of mice to virus administered by the commonly used i.n. route or to ts virus aerosolized as small particles, which are deposited primarily in the lower respiratory tract, and to large-particle aerosols (LPA), which are preferentially impacted on the mucous membranes of the upper airways (22). The serological and immune responses of the i.n.- or aerosol-vaccinated mice to the ts vaccine and to subsequent SPA challenge with a virulent homologous virus were then compared.

MATERIALS AND METHODS

Vaccine and challenge viruses. The live, attenuated, ts recombinant mutant influenza virus, labeled ts-1[E], was derived from a mixed infection of wild-type influenza A/Hong Kong/1968 (H3N2) virus and influenza A/Great Lakes/1965 (H2N2) ts-1 mutant (10) in primary bovine kidney cells. The recombinant virus, with a restrictive growth temperature of 38 C, has the ts genetic defect obtained from the H2N2 virus but contains the H3 surface antigen of the A/Hong Kong/1968 influenza virus. In our laboratory, the ts-1[E] mutant was passed twice in 10- to 12-day embryonated chicken eggs, which were incubated at 32 C for 48 h for each passage. Penicillin (250 U/ml) and streptomycin (250 μg/ml) were added

to the harvested allantoic fluid from passage 2 and maintained at −60 C in vials. The medium egg infectious dose (EID_{50}) was $10^{7.3}$/ml, with a hemagglutinin titer of 1:64. The H3 and ts characteristics of the second egg-passed ts-1[E] preparation were confirmed by B. R. Murphy (National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

A virulent, mouse-adapted variant of type A/Aichi/2/68 (H3N2) influenza strain was used for homologous virus aerosol challenge of the vaccinated mice. Procedures for the preparation of this variant have been described previously (22). Allantoic fluid containing $10^{9.3}$ EID_{50}/ml was distributed in vials after the addition of penicillin and streptomycin and frozen at −60 C. The mouse-adapted virus contained the H3N2 surface antigens of the parent strain based on analyses performed at the World Health Organization, International Influenza Center, Center for Disease Control, Atlanta, Ga.

Experimental animals. Six-week-old mice, Crl:COBS^{r}CD^{R}-1(ICR), were used in this study. After vaccination or challenge, mice were maintained in polycarbonate cages capped with filter lids. Mice receiving different treatments were segregated in ventilated biological safety cabinets. For each vaccination treatment 183 mice were used, including controls; 45 were used for determining antibody response to vaccination and 72 were used for subsequent aerosol challenge.

Aerosol dissemination, vaccination, and sampling procedures. Unanesthetized mice were exposed for 20 min to SPA (mass median diameter, 2 μm) of allantoic fluid suspensions of the ts-1[E] virus or homologous challenge virus in a modified Henderson aerosol tube apparatus (16) equipped with a Collison atomizer (8). Other groups of mice were vaccinated with ts-1[E] virus contained in LPA (mass median diameter, 8 μm) produced with a spinning-top aerosol generator (22) modified for attachment to the Henderson apparatus. Aerosol samples were collected in all-glass impingers (4) containing 20 ml of heart infusion broth. The presented, inhaled dose for mice exposed to the vaccine aerosols was computed on the basis of EID_{50}/liter of aerosol × minute respiratory volume of the mouse (0.025 liter/min) × duration of exposure in minutes (4).

i.n. vaccination. A standard volume of 0.05 ml of the ts vaccine suspension was instilled in the anterior nares of mice lightly anesthetized by ether. A control group of mice, labeled as nonvaccinated, were sham vaccinated with 0.05 ml of noninfected allantoic fluid also by the i.n. route.

Tissue titrations. To determine ts-1[E] concentrations in the upper respiratory tract (URT), the trachea and the nasopharyngeal tissues were excised from mice at specified postvaccination intervals for 27 days. Tissues from 11 mice per vaccination group were homogenized individually in Ten Broeck grinders as a 10% (wt/vol) suspension in heart infusion broth containing penicillin and streptomycin and clarified by refrigerated centrifugation. Selected dilutions of the homogenates were inoculated in eggs, using four eggs per dilution. After 48 h of incubation at 32 C, the allantoic fluid was harvested and titrated for viral hemagglutinins using chicken erythrocytes in a standard tube assay. Titration of end points were calculated by the method of Reed and Muench (14). Geometric mean titers were computed for the virus-positive mice within a vaccination group for each assay interval. Lung virus titrations for individual mice were performed on entire lungs aseptically excised at the hilus. Lung tissues were homogenized and assayed for virus content as described for the URT samples. All virus concentrations were reported as EID_{50} per tissue.

Serological assays. Serum samples, obtained from individual mice, were tested for influenza virus serum-neutralizing (SN) and hemagglutination inhibition (HI) antibodies. SN assays against 100 EID_{50} units of ts-1[E] virus were performed in eggs (15) and were considered positive if a ≥fourfold increase occurred in SN antibody. Sera for HI tests were treated with receptor-destroying enzyme (Microbiological Associates, Bethesda, Md.) and HI titers were based on standard microtiter HI tests using 4 antigen units. Bronchoalveolar wash (BAW) fluids were obtained by washing individual mouse lungs with antibiotic containing phosphate-buffered saline, pH 7.2, instilled via the trachea. Gentle aspiration of the saline two to three times in the lungs resulted in obtaining ~0.5 ml of wash fluid with no gross signs of blood. The BAW fluids of 10 mice from each treatment group were separated into two pools of five mice each to yield two replicate samples. The pooled samples were concentrated 10- to 15-fold by ultrafiltration through ~25,000-dalton filters (Millipore Corp., Bedford, Mass.) and maintained at −20 C until assayed. HI and virus-neutralizing antibody assays were performed in duplicate on replicate BAW pools as described for the serum samples. Immunoglobulin (Ig) (IgM, IgA, IgG) levels in the BAW and serum samples were measured by radial immunodiffusion assay procedures (7) as described by Palmer and Woods (13). Testing was performed using anti-mouse Ig prepared in goats and with mouse Ig reference standards prepared from ascites fluids (Meloy Laboratories, Springfield, Va.).

RESULTS

Replication of ts-1[E] virus in respiratory tissues. Infection frequency and the degree of replication of the recombinant ts-1[E] virus in the respiratory tract of the three groups of vaccinated mice were determined for 27 days after exposure (Table 1).

For the first 7 days after administration of the ts virus, virus was recovered from both upper and lower regions of the respiratory tract from most of the SPA (60 of 66) and i.n. (57 of 66) mice. A lower rate of virus recovery was obtained for the LPA-vaccinated group (29 of 66). Lung virus was isolated from only 8 of 44 of the LPA-vaccinated mice through 10 days postvaccination. The numbers of SPA (33 of 66) and i.n. (25 of 66) mice from which pulmonary virus was isolated were significantly different from the

TABLE 1. *Replication of ts-1[E] recombinant virus in respiratory tract of mice vaccinated[a] by different routes*

Determination	Day after vaccination	Lung			URT		
		SPA	i.n.	LPA	SPA	i.n.	LPA
Mouse response by respiratory tissue (no. positive/11)	1	11	11	1	10	11	7
	3	11	8	2	9	11	8
	7	9	6	3	10	10	8
	10	1	0	2	1	0	2
	14	1	0	0	0	1	0
	27	0	0	0	0	0	0
Totals		33/66	25/66	8/66[b]	30/66	33/66	25/66
Log_{10} geometric mean (± SD) virus-tissue titers of the positive mice	1	4.1 (±0.6)	6.0 (±1.1)	3.7 (±0.0)	4.4 (±0.9)	5.9 (±0.7	3.0 (±0.8)
	3	4.4 (±0.8)	4.6 (±1.2)	3.4 (±0.1)	4.1 (±0.7)	5.7 (±0.5)	4.1 (±0.9)
	7	4.3 (±0.5)	3.3 (±1.0)	3.2 (±0.8)	3.4 (±0.6)	3.1 (±0.8)	3.7 (±0.7)
	10	3.2 (±0.0)	0	2.1 (±0.2)	3.2 (±0.0)	0	2.8 (±0.4)
	14	2.3 (±0.0)	0	0	0	2.2 (±0.0)	0
	27	0	0	0	0	0	0

[a] Vaccination dose (EID_{50}) = SPA, $10^{4.1}$; LPA, $10^{4.1}$; i.n., $10^{4.7}$. SD, Standard deviation.
[b] Significantly different by Fisher's exact test ($P \leq 0.019$) from the other two lung values.

LPA group (8 of 66) by Fisher's exact test: P (SPA versus LPA) = 0.005 and P (i.n. versus LPA) = 0.019. The frequencies of virus isolation from the URT among the three vaccinated groups, however, were not significantly different. Regardless of vaccination method, practically all of the virus was cleared between 7 and 10 days, as occurs with wild-type virus infection.

Replication of the ts-1[E] virus in the respiratory tissues was less than usually seen with wild-type virus. Maximum virus titers between 10^5 to 10^6 EID_{50}/ml at days 1 and 3 were obtained in the i.n. group, whereas $10^{4.4}$ EID_{50} or less of virus per ml was isolated from the aerosol-vaccinated animals. In consonance with the 38 C shut-off temperature for this mutant, the ts virus proliferated about as well in the lungs (37 C) as in the cooler nasopharyngeal tissues (32 to 34 C). Virus attenuation was acceptable as evidenced by the lack of any pulmonary pathology or overt illness in any vaccinated mice during the 27-day observation period.

The moderate concentrations of ts virus recovered from the aerosol- and i.n.-infected mice raised the question as to whether the apparent decreased growth efficiency of this virus was due to its nonadaptation to the mouse. We, therefore, infected mice i.n. with both unadapted and mouse-adapted wild-type A2/Aichi viruses to compare these preparations with the mouse-unadapted ts mutant on the basis of respiratory tissue virus levels and infectivity. Virus titers of over 10^6 EID_{50} logs were reached in both the lungs and URT in 24 h with the two unadapted strains (ts-1[E] and wild type). In the same period the adapted virulent virus peaked at 10^8 EID_{50}. Titers of all three virus strains were at levels of $\sim 10^{5.8}$ EID_{50} through 3 days in the URT of i.n.-infected mice. The lungs had titers of $10^{4.6}$ EID_{50} at 3 days, which diminished to nondetectable levels at 10 days after infection for all three strains. Lung lesions were observed in less than 40% of the mice infected with the unadapted wild-type virus with no lethality, whereas the ts-1[E]-exposed mice had no detectable pulmonary pathology. Severe pulmonary lesions with high mortality by 10 days were recorded for mice given the mouse-adapted strain.

Antibody response of mice. The levels of virus SN and HI antibodies in the serum of vaccinated and nonvaccinated control mice are shown in Table 2. Not shown are the serum Ig levels (IgM, IgA, IgG); Ig levels were not increased over base line values for any group of mice.

SN antibodies against the ts-1[E] virus were demonstrable for all i.n. and SPA mice as early as 7 days and persisted through 27 days. SN activity for LPA was delayed until 14 days. A similar temporal pattern was obtained for serum HI antibody among the three groups of mice. More SPA mice were positive than those

in the i.n. group at 7 and 14 days. None of the LPA-vaccinated mice were positive for HI antibody at 7 days, and only three of five were positive at 14 days. The number of positive mice and HI titer levels increased to equivalent levels for the three vaccination treatments by 27 days.

The Ig responses obtained from concentrated (15×) lung wash fluids over the 27-day vaccination period are shown in Table 3. Significant increases in BAW fluid Ig levels were elicited only for IgA and IgG in the i.n.-vaccinated mice at 14 days (no measurable levels of IgM were present for any group). Positive HI titers in the BAW were detected at 14 and 27 days for i.n.-vaccinated mice and only at 27 days for the SPA group. Virus-neutralizing antibody titers of concentrated, pooled BAW fluids were higher and persisted longer in the SPA-vaccinated mice than in the i.n. groups. The BAW fluids obtained from the LPA-vaccinated mice showed no increases in Ig levels and had negative antibody responses over all assay periods.

Response of mice to virus challenge. To correlate the observed antibody responses with protection of the host, the ts-1[E] vaccinates and nonvaccinated control mice were challenged with virulent virus 28 days later. The results after SPA challenge with $10^{3.9}$ EID_{50} of a mouse-adapted, virulent variant of the Aichi influenza virus (H3N2) are shown in Table 4.

All nonvaccinated mice responded with much higher concentrations of challenge virus in both URT and lower respiratory tract tissues at 2 days after challenge than did the vaccinated groups. By 10 days the entire group had died. In contrast the i.n. and SPA group mice were totally protected, as evidenced by the lack of deaths during the 21-day observation period. In only 2 of 15 mice was virus replication detected

TABLE 4. *Response of ts-1[E]-vaccinated mice challenged at 28 days with SPA of virulent influenza A virus (H3N2)*[a]

Vaccination route	Titers of challenge virus at 2 days				Survival	
	Lung		URT			
	Positive/15	Titer (EID_{50})	Positive/15	Titer (EID_{50})	No.	%
Nonvaccinated	15	6.8[b]	15	4.9[b]	0/72	0
i.n.	2	3.5	0	0	72/72	100[c]
SPA	2	3.7	0	0	72/72	100[c]
LPA	11	5.1	6	3.8	64/72	86[d]

[a] Challenge dose, $10^{3.1}$ EID_{50} inhaled dose.
[b] Geometric mean $\log_{10}$ titer for positive animals; titers of all vaccinated groups are significantly different from the nonvaccinated group at $P < 0.005$ (t test).
[c] Significant at χ^2 ($P < 0.001$) by χ^2 with Yates correction.
[d] Significant at χ^2 ($P < 0.05$) by χ^2 with Yates correction.

TABLE 2. *Serum antibody response of mice vaccinated with the ts-1[E] mutant of influenza A virus (H3N2)*

Route of vaccination	No. of mice tested/assay	No. of mice positive					
		7[a]		14		27	
		SN[b]	HI	SN	HI	SN	HI
Nonvaccinated	5	0	0	0	0	0	0
i.n.	5	5	2 (20)[c]	5	4 (34)	5	5 (60)
SPA	5	5	4 (14)	5	5 (40)	5	5 (48)
LPA	5	0	0	5	3 (16)	5	4 (48)

[a] Days after vaccination.
[b] ≥Fourfold increase.
[c] Reciprocal geometric mean HI titer of positive mice is given in parentheses.

TABLE 3. *Ig levels and antibody titers in BAW from ts-1[E]-vaccinated mice*

Parameter	Route of vaccination											
	i.n.			SPA			LPA			Nonvaccinated		
	7[a]	14	27	7	14	27	7	14	27	7	14	27
IgA (mg/100 ml)	0	20[b]	0	0	0	0	0	0	0	0	0	0
IgG (mg/100 ml)	35	63[c]	51	<15	25	36	0	<15	32	27	32	34
HI titer (reciprocal)	0	40	40	0	0	14	0	0	0	0	0	0
Neutralization titer (reciprocal)	0	2	0	0	8	8	0	0	0	0	0	0

[a] Days after vaccination.
[b] $P < 0.001$ (t test) compared to zero values at 14 days.
[c] $P < 0.05$ (t test) compared to other assay values within and among treatments.

in the lung of SPA- or i.n.-vaccinated mice, and the lung titers were significantly lower than the nonvaccinated controls. None of the URT tissues from these two groups of vaccinated mice had detectable challenge virus. Decreased respiratory tract infectivity for the LPA-vaccinated mice also was evidenced. Although many LPA-vaccinated mice had virulent virus present in the lungs (11/15) and URT (6/15), the virus titers still were significantly lower than those measured for the nonvaccinated groups. The protection afforded against lethality for the LPA-vaccinated mice was substantial but not complete. The survival rate of 89% obtained for this group was significantly lower ($\chi^2 = P < 0.05$) than the 100% in the other vaccinated groups.

DISCUSSION

The prophylactic efficacy of attenuated, ts-1[E] recombinant influenza virus (H3N2) administered i.n. to man has been demonstrated previously (10, 11). The present studies have provided for the first time new information on the ts virus growth characteristics in respiratory tissues when given to mice i.n. and as SPA and LPA. In addition, the mouse as an experimental host provided us with a means of correlating virus replication in different regions of the respiratory tract with some indicators of immunity (e.g., BAW fluid antibodies) that are difficult to study in man and, very importantly, of correlating these immune responses with protection against lethal challenges of virulent homologous virus.

Our data revealed that the levels and clearance of the ts-1[E] virus in the SPA- and i.n.-vaccinated groups paralleled the growth pattern in mice infected i.n. with wild-type influenza A virus strains. It appears, therefore, that the growth pattern of the ts recombinant is a reflection of nonadaptation to the host and not to the ts defect. The virus clearance data imply that a carrier state probably would not be induced by vaccination with the ts-1[E] mutant. It also is noted that we report lung virus titers that are higher than those previously reported (9) for another ts-1 mutant. In the prior study, however, the authors used a different recombinant mutant (ts-1, H2N2, 38 C shut-off) for infecting hamsters and estimated virus recoveries over all animals. In our study, tissue virus levels were computed only for animals positive for virus (Table 1).

The possibility that the magnitude of the ts virus recovery levels in the lungs may have been due to reverted virus seems unlikely. Samples of virus isolates obtained from the lungs of mice in our study showed restrictive proliferation at 37 to 38 C but not at 32 C, indicating a retention of the ts defect after replication in the mouse lung. In addition, Murphy et al. (10, 11) have reported that the ts-1[E] virus was genetically stable and the ts phenotype of this recombinant was retained regardless of the host in which the virus was grown (tissue culture, hamsters, eggs, or respiratory tract of man).

The degree of virus replication in the i.n.- and SPA-vaccinated mice also reflected the similar virus deposition characteristics in the pulmonary tree with these two methods of administration. Influenza virus deposition studies in our laboratory have shown that i.n. instillation of large volumes of inoculum (0.05 ml) in anesthetized mice results in the deposit of most of the virus in their lungs (5). Larson et al. have shown also that with SPA exposure of nonanesthetized mice, 70% of the recoverable virus immediately after aerosol exposure was found in the lung; the remainder was recovered from the mucous membranes of the URT. By 24 h the virus in the upper airways usually will have proliferated to concentration levels similar to those in the lung. In mice exposed to the LPA, where practically all of the virus is retained in the URT (22), lower rates of respiratory tract infection were obtained than with SPA- or i.n.-vaccinated mice. The LPA results agreed with published data that infectivity of aerosolized organisms decreases as aerosol particle size increases (2).

Our results also indicated that the overall immune response observed in the mice appeared related to the particle size of the aerosol-administered virus. SN and HI antibodies were elicited in the i.n. and SPA mice by 7 days after vaccination. The LPA group of mice had no detectable serum titers until 14 days; moreover, the proportion of positive mice was less and the HI titers were lower in the LPA group than in the other two groups. The delayed onset of serum antibody production in the LPA mice was consistent with the concept that live virus, deposited as large particles in the upper airways, must replicate to high concentrations before seeding of the lower respiratory tract can occur. The LPA mice also differed from the other two groups in the lack of demonstrable HI and virus-neutralizing antibodies in bronchoalveolar secretions.

Whereas serum antibody levels were similar for the SPA and i.n. groups, the latter mice showed earlier production and higher titers of HI antibody in the BAW fluids than SPA mice. However, virus-neutralizing titers of concen-

trated BAW fluids were higher and persisted longer in SPA-vaccinated mice than in the i.n. group. In addition, in another study by us, mice infected i.n. with a sublethal dose of mouse-adapted wild-type A2/Aichi virus responded with virus-neutralizing antibody and HI antibodies in BAW fluids 14 to 28 days after infection and were totally protected when rechallenged (J. V. Jemski, unpublished data). The production of virus-neutralizing antibody in the bronchopulmonary washes and sera of guinea pigs given nose drops of inactivated influenza virus vaccine also has been reported (21).

Only the i.n. animals responded with significantly increased IgG and IgA levels in the BAW fluids, and this occurred at 14 days. Whether the negative Ig response for the SPA mice was due to the predominantly alveolar deposition of the virus antigen is not known. According to Tomasi (19), Ig-containing cells are not present in this region. On the other hand, the i.n.-instilled antigen probably flows down the trachea and bronchial mucosa for final deposition in the proximity of the hilar region of the lung. Local antibody-producing cells are located in the mucous lining of this pathway.

The present studies further substantiated the effectiveness of the attenuated ts-1[E] recombinant virus used as a vaccine to protect against influenza infection and mortality. All vaccinated mice were subsequently challenged with aerosols of a mouse-adapted, virulent wild-type influenza (H3N2) virus contained in small particles (≤ 5 μm). We consider this a more natural challenge than i.n. challenge. In nature, inhaled particles of this size are desposited deep in the pulmonary spaces. Not only were there no deaths in the SPA- and i.n.-vaccinated groups, but lung and URT infections after rechallenge were minimal; only 2 of 15 of the i.n.- and SPA-vaccinated mice had any virus in the lungs and at low levels. URT virus could not be detected in any of these mice. In fact, the antibody response of the i.n. mice and the total protection afforded the i.n. and SPA groups were identical to the immunity and protection observed in rechallenged mice after recovery from earlier i.n. sublethal active infections with virulent virus (J. V. Jemski, unpublished data).

The specific immune mechanism(s) for the suppression of challenge virus in both regions of the respiratory tract cannot be fully explained from the available data. Even the extent of the contribution of the circulating antibodies to the protection of the animals is not known. It is of interest to note the decreased infectivity of the challenge virus in the respiratory tract of LPA-vaccinated mice in which local HI and neutralizing antibody could not be detected but which had similarly induced serum antibodies at 27 days as the SPA and i.n. groups. Further, we cannot exclude the potential role of locally produced cellular immunity engendered by aerosol vaccination (20), as this factor was not evaluated in the current investigation.

In conclusion, we have shown that the ts-1[E] virus is cleared from the respiratory tract of aerosol- and i.n.-vaccinated mice in the same period of time (8 to 10 days) as usually recorded for wild-type influenza virus. Further, the effectiveness of vaccination with SPA of the attenuated ts-1[E] recombinant influenza virus strain has been demonstrated. It is suggested that vaccination with SPA of a ts-attenuated, replicating antigen provides a type of immunity similar to that resulting from active infection but without the clinical sequelae. In addition, the potential of mass immunization of high-risk groups to influenza with such a system appears to be an attractive and reasonable possibility.

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