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Influenza Infection in the Infant Mouse

PETER D. REUMAN,[27] ELIA M. AYOUB, AND PARKER A. SMALL, JR.

Departments of Pediatrics, and Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, Florida, USA

Summary

A nonlethal influenza infection [A/PC/1/73 (H_3N_2)] was given to infant mice to determine (1) the pathology of tracheal epithelium and lung, (2) the time course of viral shedding from the nose and lung, and (3) the subsequent development of protective immunity during adulthood.

Both desquamation of the tracheal epithelium and lung pathology similar to that described in adults after influenza infection were observed in the infant. Animals infected at 3 days of age show virus shedding in 12 of 13 infant mice that persists for at least 2 days longer than in the adult. This longer duration of influenza infection did not result from either malnutrition or from intralitter transmission of virus. Recovery from virus shedding in both the upper and lower airway occurred in the absence of detectable serum antibody in six of seven mice. Infants that recover from infection, when rechallenged during adulthood, manifest complete protection in 11 of 13 mice after nonlethal challenge and no mortality after lethal challenge.

Abbreviations

EID_{50}, egg infectious dose 50%
MID_{50}, mouse infectious dose 50%
MLD_{50}, mouse lethal dose 50%
NK, natural killer

Influenza infection is responsible for producing significant morbidity and mortality in the human infant (4, 14). The pathology of influenza disease in the adult mouse corresponds to influenza in human adults (22). This suggests that the young mouse might be a good model for studying factors responsible for the severity of influenza in the infant human. Although studies of lethal influenza infection of infant animals have been reported (3, 5, 12, 19, 23), the suitability of the infant mouse as a model for nonlethal influenza infection in the newborn has not been reported. We therefore undertook the present study to determine (1) the pathology of influenza infection, (2) recovery from influenza virus shedding, and (3) the development of subsequent protective immunity against influenza in the infant mouse.

MATERIALS AND METHODS

Animals. Eight to ten-week-old, inbred Swiss white mice of the A/J strain were obtained from Jackson Laboratories, Bar Harbor, ME. These animals were housed and bred in the animal facilities of the College of Medicine, University of Florida. All experimental and control mice were housed in the same environment and treated similarly.

Breeding. Eight female mice were housed per cage and separated from male mice for at least 1 wk before breeding. At the time of mating no more than two female mice were placed in each cage containing one male. Males were removed at the end of 4 days. Two weeks later, pregnant females were isolated in individual cages. Approximately 20% of mothers would deliver on each of 2 days, 21 days after the 4-day breeding period.

Virus. A/Port Chalmers/1/73 (A/PC/1/73 H_3N_2) influenza virus was originally obtained from the Research Resources Branch, National Institutes of Allergy and Infectious Disease. This was passaged multiple times in eggs to provide a large stock of virus that contained $10^{8.3}$ EID_{50}/ml. This virus was also passaged six times in mouse lungs to provide a suspension of the triturated lungs of infected mice in sterile phosphate buffered saline, pH 7.2, containing $10^{5.5}$ EID_{50}/ml of A/PC/1/73 (H_3N_2).

Infection. Mice were inoculated intranasally while awake or when anesthetized with sodium pentobarbitol (0.06 mg/g body weight). Mice given this amount of nembutal were unresponsive to painful stimuli. All mice were inoculated awake unless otherwise indicated. Influenza virus dosages given to infant mice are depicted in Table 1. The mortality in the 3-day-old infant mouse varied with the volume of virus solution. This is consistent with the observation in adult mice (22) and probably represents aspiration of virus into the lung with the larger volume, and restriction of initial infection to the upper respiratory tract with the smaller volume.

Virus isolation and titration. Mice were anesthetized, exsanguinated and swabbed with ethanol. Animals were opened ventrally, along the midline, from the xiphoid process to the point of the chin. The lungs and trachea were removed aseptically and placed in a sterile Petri dish. The lungs were separated from the trachea at the carina and homogenized in 1 ml of sterile phosphate buffered saline. The tracheas were bisected longitudinally and placed in fixative for microscopic studies. The methodology for virus detection using embryonated chicken eggs has been detailed elsewhere (25).

Two methods, nasal wash and nasal homogenization, were utilized for determining virus shedding from the nose. Nasal washes were performed as previously described (15). Isolation of virus by the nasal homogenization method was performed by decapitating the animal, dissecting out the nasal cavity and homogenizing it in 1 ml of sterile phosphate buffered saline.

The technique of homogenizing nasal tissue resulted in an increased amount of virus recovered from nasal samples compared to the technique of nasal washing. One day after intranasal infection with 15 μ l of suspension containing $10^{4.3}$ EID_{50}/ml of influenza virus, virus was recovered more frequently after nasal homogenization (11/11) than after nasal wash (6/11) ($P < 0.05$). Nasal homogenization also gave statistically higher viral titers when both techniques were performed on the same *adult* noses (nasal homogenization, 4.8 ± 0.2 $\log_{10}$ EID_{50}/ml and nasal wash, 3.2 ± 0.3 $\log_{10}$ EID_{50}/ml, $P < 0.001$).

Serologic testing. Hemagglutination inhibition assays were performed as previously outlined (1). Treatment with receptor destroying enzyme (13) was not performed. Briefly, the sera were first adsorbed with kaolin and chick red blood cells and heated at 56°C for 30 min.

Scanning electron microscopy. Scanning electron microscopy studies of tracheal epithelium were performed as previously described (15).

Lung pathology. Lungs of mice dying from lethal influenza infection were isolated as described above and fixed by infusing 2.5% gluteraldehyde into the trachea. Paraffin tissue mounts were made, sectioned, and slides stained with hematoxylin and eosin.

Statistical analysis. Viral and antibody titers were compared

using the t test (10) and mortality was compared using the two tailed Fisher exact test (19). For the purposes of statistical analysis, $\log_{10}$ of undetectable amounts of virus was defined as -1.

RESULTS

Newborn mice were divided into two groups and observed until they reached the ages of 3 days or 12 days. Infants of both age groups as well as adult controls were infected with influenza A Port Chalmers/1/73 (H_3N_2) virus via the intranasal route. Mice were sacrificed and their nasal cavities, trachea, and lungs were removed for pathologic study and for virus isolation.

Morphologic changes in the epithelial lining of the trachea: studies by scanning electron microscopy of trachea. Five days after nonlethal infection with egg passaged influenza A/PC/1/73 (H_3N_2) virus mice were sacrificed and their tracheal epithelium studied by scanning electron microscopy. Desquamation occurs after infection, with changes in the infant being similar to those encountered in adult animals (Fig. 1). It was difficult to quantitate tracheal desquamation. The duration of desquamation appeared to be prolonged in the mouse infected at 3 days of age compared to the adult mouse.

Table 1. *Virus dosages used for experimental protocol*

	Mouse age	Anesthesia	Amount of virus: μl	Amount of virus: EID_{50}
Egg passaged influenza[1]	Infant	No	5	100
Nonlethal injection	Adult	No	30	300
Sixth passaged influenza[2]	Infant	No	25	$10^{4.5}$
Lethal injection	Adult	Yes	50	$10^{5.5}$

[1] An egg passaged influenza virus pool ($10^{8.3}$ EID_{50}/ml), which showed the following characteristics in: *(a)* 3-day-old infant mice not given anesthesia: mouse infectious dose 50% = 10 EID_{50} in 5 μl; mortality = 300 EID_{50} in 5 μl gave 0% mortality and 300 EID_{50} in 15 μl gave 75% mortality and *(b)* adult mouse not given anesthesia: mouse infectious dose 50% = 10 EID_{50} in 30 μl; mortality = $10^{6.8}$ EID_{50} in 30 μl gave 0% mortality.

[2] Sixth mouse passaged influenza virus pool ($10^{5.5}$ EID_{50}/ml), which showed the following characteristics in the adult mouse given anesthesia: mouse infectious dose 50% = $10^{1.2}$ EID_{50} in 50 μl and mouse lethal dose 50% = $10^{3.2}$ EID_{50} in 50 μl.

Pathology of lung specimens. Infant and adult mice were given a lethal dose of influenza A/PC/1/73 (H_3N_2) virus and examined for death every 8 hr. As has previously been observed by others (5), infant mice died earlier, at 4–5 days after infection, compared to virgin adult mice that died at 8–9 days after infection. Typical sections of lung pathology found after death in both groups are presented in Figure 2. Lung pathology was characteristic of influenza pneumonia. More damage and more lymphocytes per area of lung were found in adult lung sections, probably because of the longer duration of infection. The lung pathology was otherwise similar in both the infant and adult mouse.

Time course of viral shedding. Groups of 3- and 12-day-old infant mice and control adult mice were given a nonlethal dosage of egg passaged influenza A/PC/1/73 (H_3N_2) virus and analyzed for virus shedding on various days after infection (Fig. 3). All infant mice showed evidence for recovery from infection. Virus shedding appeared to be prolonged in 3-day-old infant mice when compared to 12-day-old and adult mice.

The duration of virus shedding in the infant mouse was further examined in a separate experiment. A group of 3-day-old infant mice was infected with a nonlethal dosage of egg passaged influenza A/PC/1/73 virus at the same time as a group of adults. Eleven 3-day-old mice sacrificed the day after infection were all infected as shown by virus isolation from nasal homogenates. The virus yield from nasal wash (0.5 ± 0.7 $\log_{10}$ EID_{50}) was approximately 100-fold less than that of the adults shown in Figure 3 on day 1. Some of this difference may be attributable to the difference in nasal surface area. Thirteen days after being infected, when infants and adults were compared, the fraction of mice positive for virus was significantly different (nose, 10/13 infants *versus* 0/9 adults, $P < 0.001$ and combined nose and lung, 12/13 infants *versus* 0/9 adults, $P < 0.001$); thus, when the 3-day-old infant mouse is infected, virus shedding is prolonged to 13–14 days after infection. This is approximately 4–5 days beyond viral shedding in the adult mouse (Fig. 3).

Factors shown not to contribute to prolonged virus shedding in the infant mouse. (1) Nutritional status as reflected by weight gain. Infant mice were weighed sequentially after infection to determine if influenza infection led to malnutrition (data not shown). The weight gain for the infected groups remained within 10% of the control groups and the weights of both groups were always above the 60th percentile of the standard weight gain for A strain mice

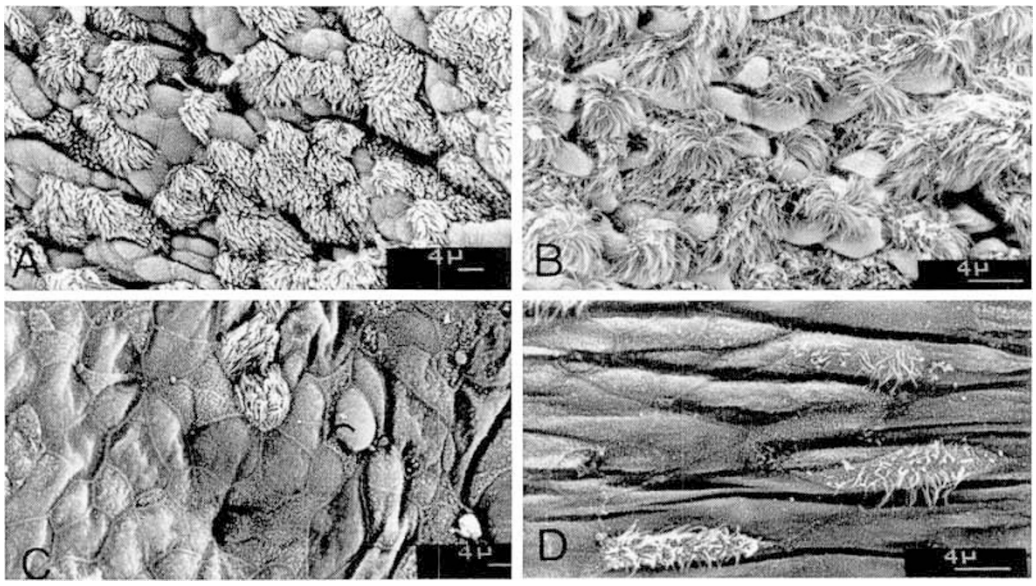

Fig. 1. The tracheal surface of a normal 3-day-old mouse (A) and a normal adult mouse (B). The mucosa of both A and B are covered with ciliated and serous cells. The tracheal surface of a 3-day-old (C) and adult mouse (D) 5 days after viral infection. Both ciliated and serous cells have been desquamated, exposing the basal layer of cells.

(6). *(2) Intralitter transmission of virus.* The prolongation of virus shedding in the infant mouse might be secondary to the transmission of virus within individual litters (18). Because the infant mouse lacks IgA (11), which is probably responsible for the prevention of reinfection, intralitter transmission of virus could be responsible for prolonged virus shedding. To determine this, we performed the following experiment. All litters were culled of infants leaving only one infant per mother. These mother-infant pairs were housed separately and divided into two groups. In the first group, both infant and mother were infected simultaneously; in the second group only the 3-day-old infants were infected. One or 13 days after infection, infants were sacrificed, the nasal cavities washed, the lungs isolated and homogenized for quantitation of virus and the serum titered for antibody. As seen in Table 2, Group 1, and in confirmation of the earlier results, all five infected mothers had stopped shedding virus by day 13 but all five surviving infants were still shedding virus. When the surviving infants nursed by infected mothers (Group 1) were compared with surviving infants of uninfected mothers (Group 2), there was no significant difference between these two groups of infants in either the number shedding virus or the amount of virus shed at day 13. Similarly, when virus shedding from single infants of uninfected mothers was compared to virus shedding from infants of larger litters, no statistical differences were found. Intralitter transmission of virus either from mother to infant or from infant to infant, cannot account for the prolonged virus shedding observed when 3-day-old mice are infected with influenza virus.

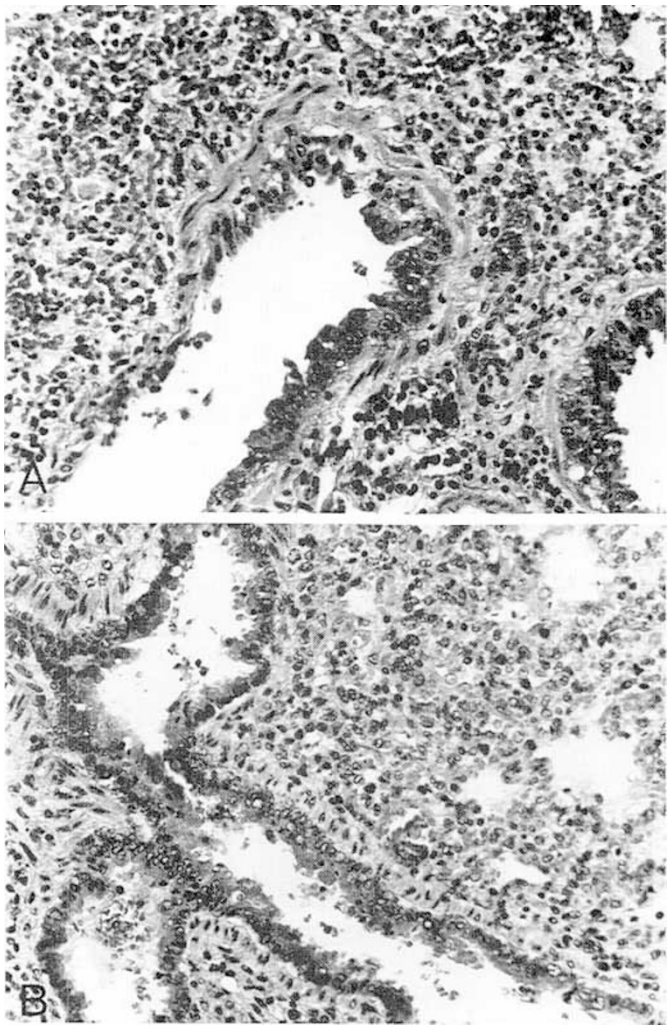

Fig. 2. The lung pathology within 8 hr of death after intrapulmonary influenza virus challenge: (*A*) adult mouse and (*B*) the 3-day-old infant mouse.

Susceptibility to infection of previously infected infant mice upon rechallenge as adults. Mice that recovered from an initial challenge as infants, were rechallenged with 10 MID_{50} of the egg passaged influenza virus pool 5–10 wk later (Table 3, Group 1). No virus could be isolated on rechallenge from any of these animals, whereas, virus was isolated from all adult control animals; thus, an initial intranasal influenza infection in the mouse at 3 days of age, will stimulate immunity that will prevent reinfection with homologous virus on rechallenge.

The second group in Table 3 documents results after rechallenge with 10^3 MID_{50} of the egg passaged influenza virus pool. Results after this rechallenge dosage do not show a significant difference in the fraction of mice positive for virus ($P = 0.36$) between adult mice initially challenged during infancy and the adult mice initially challenged during adulthood. Again we find no evidence for any difference in the immunity stimulated after infection in the infant when compared to the immunity stimulated in the adult.

The last group in Table 3 depicts the results found after rechallenge with 10 MLD_{50} of the sixth passaged influenza virus pool given intranasally to anesthetized adult mice. All mice sacrificed 1 day after this challenge were found to be shedding virus from the upper and lower airway. In spite of this initial shedding, no mortality occurred in any of the animals with previous exposure to influenza virus. Control adults, however, showed 100% mortal-

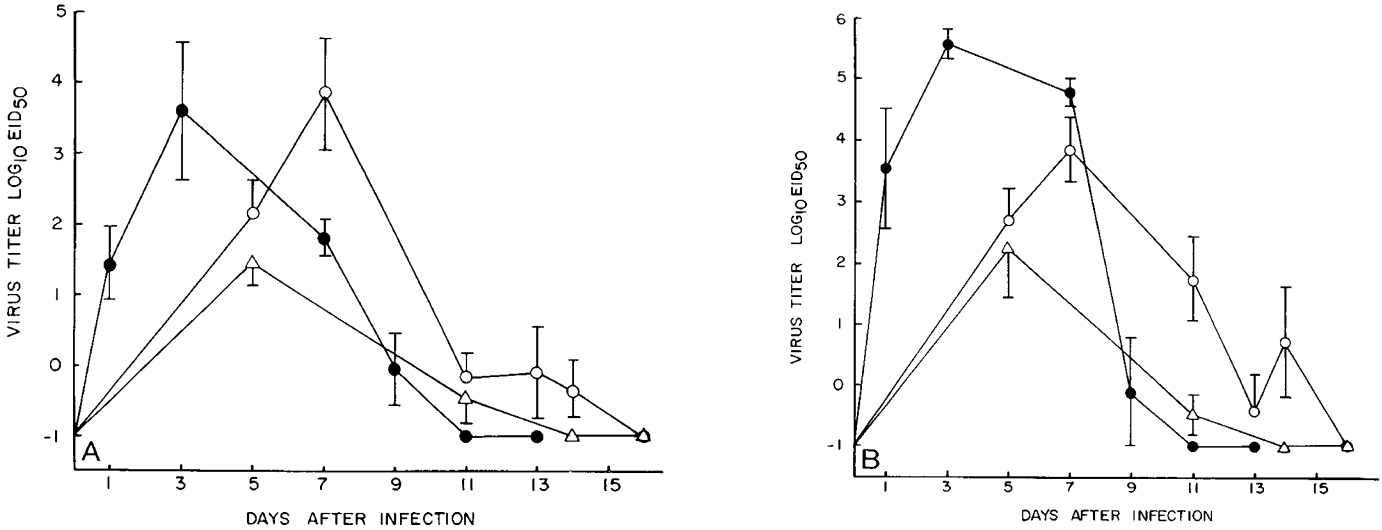

Fig. 3. A comparison of A/PC/1/73 (H_3N_2) influenza virus shedding from the noses, *left hand panel*, and lungs, *right hand panel*, of mice infected at 3 days (0), at 12 days (△) and as 8-wk-old adults (●). Three to eight animals were sacrificed each day. Nasal virus titers were obtained by the nasal wash technique. The *bars* represent standard errors of the mean. Undetectable levels of virus are represented by $\log_{10} = -1$.

Table 2. *Effect of intralitter transmission of virus on duration of infection in the infant mouse*[1]

	Fraction with positive titer			Titer (LOG_{10} EID_{50} or HI) Day 13	
Group	Day 1	Day 13		Mean	S.E.
Mother and infant infected					
Infant					
Nasal virus	6/6	4/5	5/5	−0.17[4]	0.26
Lung virus	6/6	2/5		−0.626[5]	0.25
Serum HI	0/2	2/5		(3/5 < 1:8; one 1:8 and one 1:16)	
Total infected[6]	6/6	5/5[3]			
Fraction dying before day 13		10/15[2]			
Mother					
Nasal virus	4/4	0/5			
Lung virus	4/4	0/5			
Serum HI	0/4	5/5		1:32	(1:16–1:64)
Total infected	4/4	5/5			
Infant only infected					
Infant					
Nasal virus	5/5	5/7	6/7	0.076[4]	0.37
Lung virus	4/5	3/7		−0.42[5]	0.32
Serum HI	0/3	1/5[6]		(4/5 < 1:8; 1 = 1:16)	
Total infected	5/5	6/7[3]			
Fraction dying before day 13		27/35[2]			
Mother					
Nasal virus		0/7			
Lung virus		0/7			
Serum HI		0/7		All < 1:8	
Total infected		0/7			

[1] Two groups of single infant litters were infected with A/PC/1/73 (H_3N_2) influenza virus. In the second group, one mother was positive for lung virus and antibody. This mother and her infant have been dropped from the analysis. Results of groups 1 and 2 are compared as noted by paired footnotes:

[2] Fischer's exact test, $P = 0.20$.

[3] Fischer's exact test, $P = 0.58$.

[4] Student's t test, $P = 0.64$.

[5] Student's t test, $P = 0.64$.

[6] Two sera were of insufficient quantity.

[7] As evidenced by virus isolation from nose and/or lung.

Table 3. *Influenza immunity on adult rechallenge after initial infection in the infant mouse*[1]

	Initial infection		Adult rechallenge				
			Serum antibody titer				
Rechallenge group	Age	Dosage[2] (Mouse ID_{50})	Age (wk)	$Log_2 \pm$ S.E.	Dosage (Mouse ID_{50})	Fraction positive for virus[4]	Fraction dying[5]
Group 1, Low dosage nonlethal egg passaged virus given to awake adult	3 day	$10^{1.0}$	5		$10^{1.0}$	0/8	
	6 wk	$10^{1.3}$	16		$10^{1.0}$	0/3	
	Control		8		$10^{1.0}$	5/5	
Group 2, High dosage nonlethal egg passaged virus given to awake adult	2 day	$10^{1.0}$	14	5.4 ± 0.4 (5)[3]	10^{3}	2/5	
	8 wk	$10^{1.6}$	20	6.2 ± 0.6 (6)[3]	10^{3}	0/6	
	Control		12–14		10^{3}	7/7	
Group 3, High dose lethal 6th passaged virus given after anesthetizing adult	2 day	$10^{1.0}$	10	4.0 ± 0.0 (2)[3]	$10^{3.2}$	4/4	0/4
	8 wk	$10^{4.9}$	18	6.0 ± 0.3 (5)[3]	$10^{3.2}$	5/5	0/5
	Control		10		$10^{3.2}$	5/5	5/5

[1] Infant and adult mice were initially infected with a nonlethal dosage of A/PC/1/73 (H_3N_2) influenza virus and rechallenged 5–14 wk later with three separate A/PC/1/73 viruses differing in dosage and passage characteristics.

[2] Intranasal without anesthesia; Mouse ID_{50} vary with age at time infected.

[3] Number of animals titered in parentheses.

[4] Fraction positive for virus in nose and/or lung one day after rechallenge for groups 2 and 3 and 3 days after rechallenge for group 1.

[5] Death did not occur in groups 1 and 3 because animals were not anesthetized (30).

ity. It should also be pointed out that the adult mice of groups 2 and 3, rechallenged 18–20 wk after their initial infection, differ significantly in the fraction positive for virus. This difference was present despite approximately equal rechallenge dosages and was accounted for either by the differences in passage characteristics of their initial viral infection or by the differences of anesthesia given to the adults of Group 3 before their initial infection. Irrespective of this difference, the above results indicate that

immunity acquired after infection protects against lethal rechallenge when the infant is initially infected as early as 2 days of age.

DISCUSSION

In this study we examined (1) pathology, (2) virus shedding, and (3) subsequent immunity after influenza virus infection in the infant mouse. Influenza-induced tracheal desquamation was shown to occur in the infant mouse 1 day after the onset of infection in the first wk of life. The normal infant mouse is known to be Tc deficient for 6–9 days (24), B-cell deficient for 1 wk (11) and NK-cell deficient for 3–4 wk (7). Although influenza infection may alter this immune development, it seems unlikely to be able to do so rapidly enough to affect events during the first day of infection like tracheal desquamation. Our findings suggest that Tc, NK, and B cells do not play a role in producing tracheal desquamation. Influenza-induced tracheal desquamation, therefore, appears to be a virally mediated phenomenon, which is independent of B, T, and NK cell function.

Infant mice had a shortened survival time but showed less severe lung pathology and fewer lymphocytes per area of lung at the time of death when compared to the adult. Our findings concerning shortened survival and greater mortality in the infant mouse are consistent with previous studies utilizing lethal challenge (3, 5, 19, 23). The more severe influenza disease in the infant mouse may be attributable to viral dissemination (5), immune deficiency, or other untested factors.

The results of our study revealed that nonlethal influenza virus infection in the 3-day-old infant mouse resulted in delayed recovery as evidenced by virus shedding from the nose and lung for 4–5 days longer than in the adult. Numerous investigators have examined the relationship between the age of a given host and its susceptibility to various infectious agents (20). Sawicki (16, 17) found prolonged shedding when paramyxovirus was given to the infant mouse. Several investigators have looked at lethal influenza infection in the young animal (3, 5, 12, 19, 23), but none have studied the characteristics of nonlethal influenza infection in the young animal.

Delayed recovery in the infant mouse might be attributed to several variables, including (1) transmission of virus within each litter, (2) malnutrition secondary to infant infection, and (3) developmental differences of the infant mouse. Intralitter transmission occurs (18) and theoretically could result in reinfection in animals lacking IgA (11), the probably primary preventive immune mechanism; however, intralitter transmission was shown not to contribute to prolonged virus shedding in the infant mouse. Without evidence for severe malnutrition and its coincident immune deficiencies (2, 8), it is unlikely that small differences in weight gain between infected and control groups contribute to delayed recovery; thus, both intralitter transmission and malnutrition were found not to contribute to delayed recovery.

Prolonged virus shedding of the infant compared to the adult mouse appears to be related to developmental differences. Although these differences may be solely a factor of the infant's known immune deficiencies, other factors cannot be ruled out. Different viral replication kinetics as suggested by the later peak virus titers of younger animals, may be involved in prolonging virus shedding. Further study is required to know which of these factors contributes to the prolonged viral shedding.

Immunity stimulated in the infant after initial nonlethal influenza infection appears to *prevent* virus replication after low dosage intranasal rechallenge and to *protect against* mortality after lethal pulmonary rechallenge. This immunity did not differ from that stimulated in the adult. The mechanism responsible for prevention of influenza virus replication in the lung and nose was stimulated as a result of the infant's infection. Prevention of influenza infection in the upper airway is believed to be IgA mediated (1, 9) and in the lower airway it is IgG mediated (15, 21). The onset of IgA and IgG production has been found to occur at approximately 3 wk and 2 wk of age respectively (9, 11); therefore, either the cells necessary for the functioning of these two protective immune mechanisms are stimulated in the infant during the course of virus shedding or viral antigen persists until these immune components are able to be stimulated. Once these immune mechanisms are stimulated, the mouse is protected against rechallenge during adulthood.

We have shown that influenza infection in the infant mouse results in more severe disease than in the adult mouse. The infant shows prolonged virus shedding after nonlethal infection and shortened survival after lethal infection. The differences in viral shedding between the infant and the adult may be related to differences in immunologic development. Typical influenzal tracheal desquamation and lung pathology occur in the infant mouse. Recovery from upper and lower airway influenza viral shedding was prolonged in the infant. Despite functional deficiencies of the infant mouse immune system (11), no evidence was found for deficiencies in influenza specific immunity on rechallenge during adulthood. The newborn mouse appears to be a satisfactory model for studying the relationship of the ontogeny of the immune system to respiratory viral infection. Further studies should be undertaken to examine this relationship.

If the assumption is made that influenza infection does not change the normal ontogeny of the immune system, then these findings when compared with the reported ages of onset of immune component functions (11) suggest the following conclusions: (1) that specific immunity and NK cells play no role in producing upper and lower airway influenza pathology; (2) that IgA and NK cells play no role in recovery from influenza disease; (3) that the onset of cytotoxic T cell function correlates with the ability to recover from influenza infection; and (4) that these age-dependent functional immune deficiencies do not interfere with the development of subsequent immunity to influenza.

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