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Effects of Low- and High-Passage Influenza Virus Infection in Normal and Nude Mice

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A human isolate of type A Hong Kong influenza virus (H3N2) was adapted to mice by serial passage. Lung homogenates from mice who received low passage levels contained about the same quantity of virus ($10^{6.2-6.95}$ 50% tissue culture infective doses/ml) as those from mice who received high passage levels ($10^{5.95-6.45}$ 50% tissue culture infective doses/ml); however, death occurred only in animals given high-passage virus. Passage 3 (P3) and passage 9 (P9) viruses were selected as representative of low-passage and high-passage viruses, respectively. Although minimal differences were detected in infectivity for rhesus monkey kidney tissue cultures and mice, P9 virus was at least 10,000 times more lethal for mice (mean lethal dose = $10^{4.2}$). Infection with P3 virus was accompanied by minimal bronchitis and bronchiolitis only, whereas P9-infected animals exhibited marked bronchitis, bronchiolitis, and pneumonia. Striking thymic cortical atrophy was also demonstrable in the P9-infected animals and, although virus was more commonly recovered from thymuses from these animals, immunofluorescent studies revealed only a few cells containing influenza virus antigens. To further explore the participation of thymus-derived lymphocytes in influenza, athymic nude mice and furred immunocompetent littermates were given 500 50% mouse infectious doses of P9 virus. Nude mice exhibited an increased survival time and, in contrast to the extensive lung pathology seen in furred littermates, manifested minimal cellular infiltration and no tissue destruction in lungs. Brains from nude mice exhibited encephalomalacia with lymphocytic perivascular cuffing, which was not seen in furred animals. Virus was recovered from brains of 6 of 13 nude mice and 1 of 10 furred animals. The contrasting models suggest that thymus-dependent cells play a significant role in the inflammatory response to influenza virus infection and should prove useful for probing host-virus interactions which characterize influenza virus virulence.

The development of infection and illness after exposure to influenza virus depends in large part on the host's immune status. Stimulation of the antibody-producing (B cell) system by influenza virus protects against the disease as indicated by the correlations of immunity with serum antibody levels (18, 22, 34). A requirement for a T-cell helper effect in order to elicit a protective primary antibody response after influenza infection has been demonstrated in mice (34, 35).

In addition to production of antibody after infection or vaccination with influenza virus, immunological responses in human subjects have included delayed cutaneous responses to influenza antigens (11), in vitro lymphocyte blast transformation in the presence of influenza antigens (3, 27), and lymphocyte-mediated cytotoxicity for influenza virus-infected target cells (10). Similarly, studies utilizing migration inhibition assays (6, 36, 38), lymphocyte-mediated cytotoxicity (2), and blast transformation (12) have shown responsiveness to influenza virus antigens by cells from appropriately sensitized mice and guinea pigs. The role of the T-cell sensitization suggested by these studies is uncertain. Recent work utilizing adoptive immunizations (4) and treatment with antithymocyte serum (33) suggests that the cell-mediated immunological system can, under certain conditions, increase the severity of influenza pneumonia in mice. Also, athymic nude mice were recently reported to develop prolonged disseminated influenza virus infections in contrast to the more acute disease in immunologically intact animals (32). These results

contrast to an earlier report (14) in which antilymphocyte serum failed to show any effect on influenza disease.

The mouse model has been used extensively to study the pathogenesis (13, 16–18, 31, 32) and immune response (2, 5, 6, 12, 15, 23, 34, 35, 37) to influenza viruses. When administered intranasally to mice, strains of influenza virus adapted by several passages in these animals characteristically cause an acute and often lethal pneumonia (7; R. B. Grunert, unpublished data). In contrast, lower passage levels of less well-adapted influenza virus cause limited pneumonia and minimal mortality (7; Grunert, unpublished data). By comparing the effects of low- and high-passage influenza virus in mice, two degrees of disease severity are available for evaluation of the quantitative association between lymphocyte responses and the severity of disease. This paper describes studies with low- and high-passage influenza virus in immunologically intact and athymic nude mice which suggest that thymus-derived cells contribute significantly to the pathogenesis of influenza disease.

MATERIALS AND METHODS

Mice. Young adult (19 to 25 g) BALB/c mice (Texas Inbred Mouse Co., Houston, Tex.) and nu/+ (heterozygous, furred) or nu/nu (homozygous, nude) outbred Swiss mice (Laboratory Supply Co., Indianapolis, Ind.) were used. Animals were housed in cages covered with barrier filters and fed mouse chow and water ad libitum.

Virus. The 1968 strain of type A Hong Kong influenza virus (H3N2) used in these studies was isolated from a patient experiencing influenza. The virus was passaged two times in rhesus monkey kidney tissue culture (RhMK) and then passaged serially in BALB/c mice. Passage 1 (P1) was obtained by intranasally inoculating a 0.05-ml suspension of the starting virus into each of 10 lightly anesthetized animals. After 48 h these mice were sacrificed and their lungs were removed, weighed, and teased into a suspension of Hanks balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, N.Y.) supplemented with 2% gelatin. After three freeze-thaw cycles in dry ice and acetone and a 37°C water bath, the suspension was brought to 10% (wt/vol), clarified of gross debris by centrifugation and stored in 0.5-ml portions at −70°C.

Subsequent passage levels were similarly prepared by inoculating 0.05 ml of the previous passage level into each of 10 mice.

Virus isolation and quantitation. Organs were removed aseptically, washed in sterile HBSS, homogenized with sterile sand, and suspended in 1 ml of veal infusion broth. From each suspension, 0.2 ml was added to RhMK cultures (Flow Laboratories, Rockville, Md.). Five and ten days after inoculation, each culture was tested for virus by hemadsorption (26) with guinea pig erythrocytes. Hemadsorbing cultures were spot checked for virus specificity using fluorescein isothiocyanate (FITC)-conjugated antiserum (Cappel Laboratories, Inc., Downingtown, Pa.) specific to type A influenza.

The quantity of virus in organs was determined as 50% tissue culture infectious doses ($TCID_{50}$ [1]) by inoculating 0.2 ml of decimal dilutions of organ suspensions into four tubes of RhMK cultures and testing for virus as described above. The quantity of virus that could infect (MID_{50}) or kill (MLD_{50}) 50% of inoculated mice was determined by preparing 10-fold dilutions of virus in HBSS and inoculating 0.5 ml of each dilution intranasally into groups of four animals. Six days after inoculation, mice used in the MID_{50} assay were sacrificed and their lungs were assayed for virus as described above. The number of mice surviving or dead at the end of the 10-day observation period was recorded. The $TCID_{50}$, MID_{50}, and MLD_{50} end points were calculated using the procedure of G. Karber (25).

Histological methods. Organs were prepared for histological studies by fixation in buffered formalin, embedding in low-melting-point paraffin, and sectioning at 5-μm thickness. Sections were stained with hematoxylin and eosin or Giemsa stain (19).

Fluorescent-antibody procedures. To detect influenza antigens, tissue sections from normal or influenza virus-infected animals were prepared for immunofluorescent studies using the method described by Sainte-Marie (28). These sections were overlayed for 30 min at 37°C with high-titer rabbit anti-influenza virus serum (Flow Laboratories, Rockville, Md.), washed for 10 min with saline buffered to pH 7.4, and overlayed with FITC-conjugated goat anti-rabbit gamma globulin (Cappel Laboratories).

Controls for the specificity of the fluorescent-antibody staining included tissue sections or cell suspensions treated with unlabeled normal rabbit serum before the FITC-coupled anti-rabbit serum. The specificity was also demonstrated by showing inhibition of fluorescence by unconjugated goat anti-rabbit gamma globulin before the FITC-coupled antiserum and by removal of specific antibody from antiserum by adsorption with antigen. All sections were stained with Evans blue before the addition of fluorescein-conjugated serum to eliminate background fluorescence (29) and were examined with a Leitz Wetzlar Ortholux II ultraviolet microscope using an HBO-200W mercury burner, BG 12 excitation filter, and 470 or 540 barrier filter.

Cell counting and identification. Enumeration of cells in suspension was made using a Coulter counter (model ZBI, Coulter Electronics, Inc., Hialeah, Fla.). Zap-isoton (Coulter Diagnostics, Hialeah, Fla.) was used to lyse erythrocytes before each count.

RESULTS

Mouse adaptation. Early passage levels (P1 through P3) of influenza A Hong Kong virus were not lethal for BALB/c mice (Table 1). Although some animals inoculated with P4 and P5 died, it was only after P5 that the virus had the capacity to cause death consistently in 75 to 100% of the mice inoculated. Titration in tissue culture of the 10% (wt/vol) lung suspension comprising P1 yielded $10^{3.95}$ $TCID_{50}$/ml. All sub-

TABLE 1. *Adaption of human Hong Kong (H_3N_2) influenza virus to mice*

Mouse passage no.	10% Suspension of lung collected at 48 h	
	$TCID_{50}$/ml (log_{10})	Lethality for test mice
1	3.95	0/4[a]
2	6.20	0/4
3	6.95	0/4
4	6.20	1/4
5	6.20	2/4
6	6.45	3/4
7	5.95	4/4
8	6.20	3/4
9	6.20	3/4
10	6.45	3/4
11	6.20	3/4

[a] Number of mice that died during a 10-day observation period/number of mice inoculated.

sequent mouse passage lung suspensions contained $10^{5.95}$ to $10^{6.95}$ $TCID_{50}$ of virus per ml, indicating that adaption of this virus to the mouse was complete (24, 25).

Characterization of P3 and P9 virus. P3 and P9 lung suspension virus pools were selected for further characterization as representatives of low- and high-passage influenza viruses. P3 and P9 virus had similar infectivity titers both for BALB/c mice and for monkey kidney tissue culture cells (Table 2). However, infection of mice with P3 virus caused no deaths even when the inoculum contained a dose of greater than 100,000 MID_{50}. In contrast, the P9 virus pool had a titer of greater than 10,000 MLD_{50}/ml, and one 50% lethal dose was equivalent to approximately 50 MID_{50}.

Virus recovery rates from the lungs, brains, spleens, and thymuses of BALB/c mice were determined after intranasal inoculation with 100 MID_{50} of each of the viruses. Virus was recovered from the lungs of 93% or more of mice tested 6 days after inoculation with either virus (Table 3). With rare exception, neither virus was recovered from brains nor from spleens of mice inoculated with P9. However, differences in recovery rates of virus from thymuses were encountered. Virus was recovered from thymuses of 6 to 14 (43%) P3-inoculated mice in contrast to 44 of 56 (79%) mice inoculated with the P9 virus ($\chi^2 = 5.36$, $P < 0.025$).

Effects of P9 virus on nude mice. Nude Swiss mice (9) and their furred littermates were inoculated intranasally with equivalent doses (500 MID_{50}) of P9 virus. Less than half of the immunocompetent, thymus-bearing mice survived 4 days after inoculation, and only 1 of 20 survived from day 7 onward (Fig. 1). In contrast, athymic (24) nude mice had a more gradual decrease in survival, with 50% remaining 7 days after inoculation and 20% at the end of the 10-day observation period. The remaining nude mice had persistent infection when sacrificed at 14 days.

At the time of death, nude mice had somewhat higher lung virus titers than furred mice (mean, $10^{6.3}$ versus $10^{5.4}$ $TCID_{50}$/ml, respectively; Wilcoxon, $P < 0.02$). Brains harvested after death yielded virus from 6 of 13 nude but only 1 of 10 furred mice.

Histological findings. Daily examination of the lungs of furred animals infected with P3 virus revealed a progressive bronchitis which reached a peak on day 3 or 4 postinoculation. There was a moderate to marked peribronchial and peribronchiolar lymphocytic infiltrate, with a few scattered polymorphonuclear leukocytes and macrophages. Scattered microareas of interstitial pneumonitis were also seen. These findings were most marked 3 to 4 days postinoculation (Fig. 2). The thymuses and brains of these animals appeared normal.

Immunologically intact mice inoculated with P9 virus developed acute bronchitis and a marked interstitial pneumonitis associated with obliteration of many bronchioles by 2 days after inoculation. The interstitial and peribronchial infiltrates consisted of polymorphonuclear leukocytes, lymphocytes, and macrophages. The bronchial and bronchiolar epithelium underwent extensive necrosis and sloughing in the next 2 to 3 days. Scattered alveoli contained protein-rich fluid, polymorphonuclear leukocytes, and macrophages on day 2. This pneumonia became progressively more confluent

TABLE 2. *Comparison of the infectivity and lethality titers of P3 and P9 influenza virus suspensions*

Measurement[a] (log_{10}/ml)	Influenza virus passage	
	P3	P9
$TCID_{50}$	6.95	6.87
MID_{50}	6.80	5.80
MLD_{50}	0	4.20

[a] Abbreviations: $TCID_{50}$, 50% Tissue culture infectious doses; MID_{50}, 50% mouse infectious doses; MLD_{50}, 50% minimal lethal doses.

TABLE 3. *Recovery of influenza virus from organs of mice 6 days after inoculation with 100 MID_{50} of virus*

Virus inoculum	Organ			
	Lung	Brain	Spleen	Thymus
P3	13/14 (93)[a]	0/14 (0)	ND[b]	6/14 (43)
P9	55/56 (98)	1/22 (6)	2/56 (4)	44/56 (79)

[a] Ratios indicate the number of mice from whom the indicated organ yielded virus/number of mice tested. Numbers in parentheses are the ratios converted to percentages.

[b] ND, Not done.

and extensive over the next 2 to 4 days and was characterized by alveolar exudates containing macrophages and lymphocytes, focal collections of lymphocytes in alveolar septae, focal necrosis of alveolar septae, and focal areas of hemorrhagic pneumonia (Fig. 3). Occasional animals developed segmental or lobar pneumonia with abscess formation, thought to represent secondary bacterial pneumonia.

There was an acute decrease in the size of the thymus glands of immunologically intact mice infected with P9 virus (Fig. 4). This decrease in thymic size was paralleled by a dramatic decrease in the number of thymocytes obtainable after infection (Table 4) and by a marked decrease in cortical lymphocytes (Fig. 5 and 6). The medullary regions of the thymuses from acutely ill mice contained increased numbers of dark-staining lymphocytes, many of which packed the lymphatics (Fig. 7). In some thymuses, small focal collections of polymorphonuclear leukocytes were located beneath the fibrous capsule and in scattered locations throughout the cortex. Fluorescent-antibody localization of influenza virus antigens in sections of thymuses revealed only occasional virus-positive cells in glands from P9-infected mice. Concomitant studies of thymuses from P3-infected mice were essentially normal.

In contrast to the findings in immunologically intact mice infected with P9 virus, lungs of athymic nude mice infected with this or P3 virus had negligible lymphocytic or polymorphonuclear infiltration and no tissue disruption, even when examined up to 8 days postinoculation. However, examination of brains of

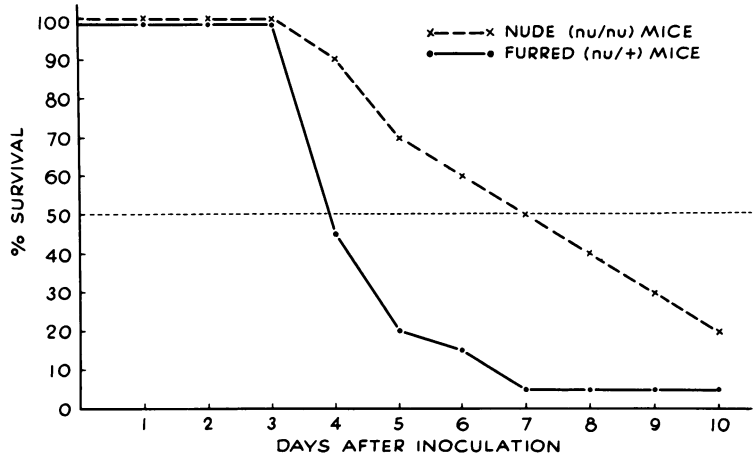

FIG. 1. *Survival of nude (nu/nu) and furred (nu/+) mice inoculated with 500 MID_{50} of high-passage (P9) virus.*

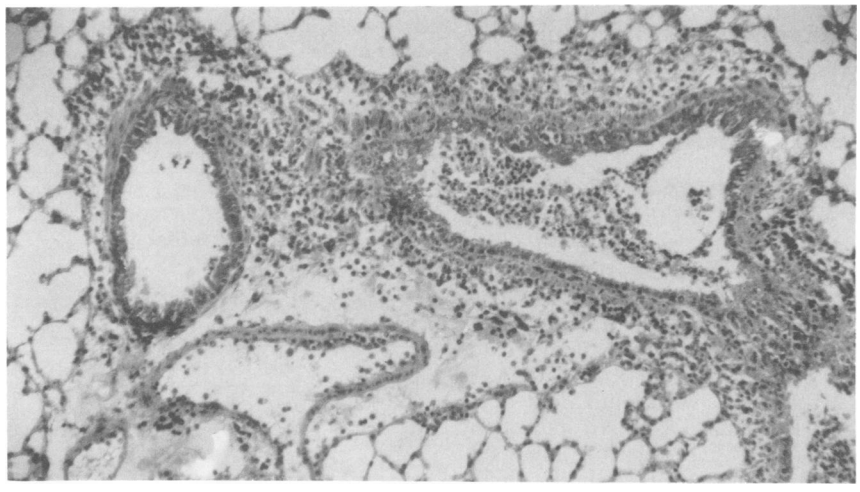

FIG. 2. *Photomicrograph of lung of BALB/c mouse 5 days postinoculation with low-passage (P3) virus. The bronchus contains desquamated epithelial cells and degenerating inflammatory cells. There is a peribronchial infiltrate but little pneumonitis. Hematoxylin and eosin stain (×50).*

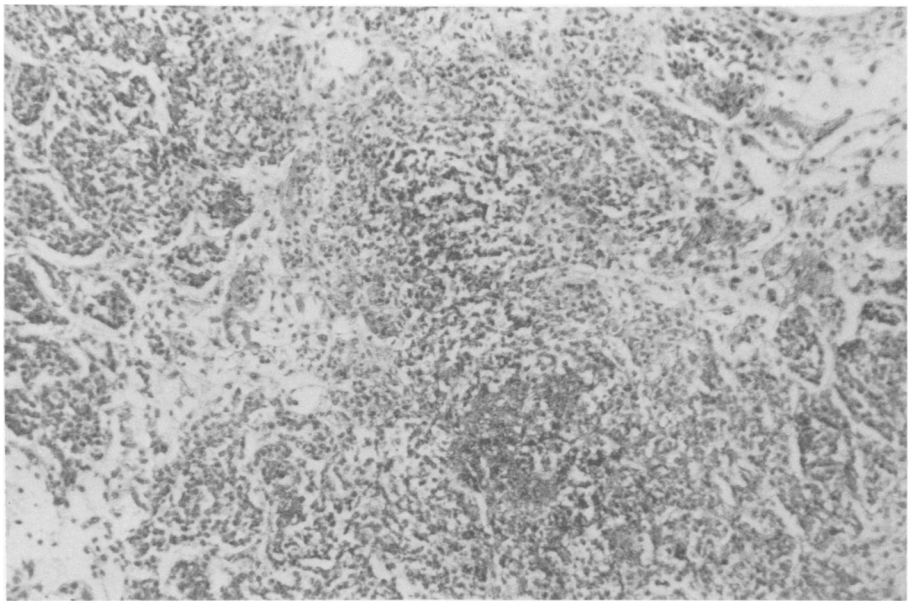

FIG. 3. *Mouse lung 5 days postinoculation with high-passage (P9) virus illustrating the complete destruction of the lung parenchyma by the inflammatory reaction. Hematoxylin and eosin stain (×50).*

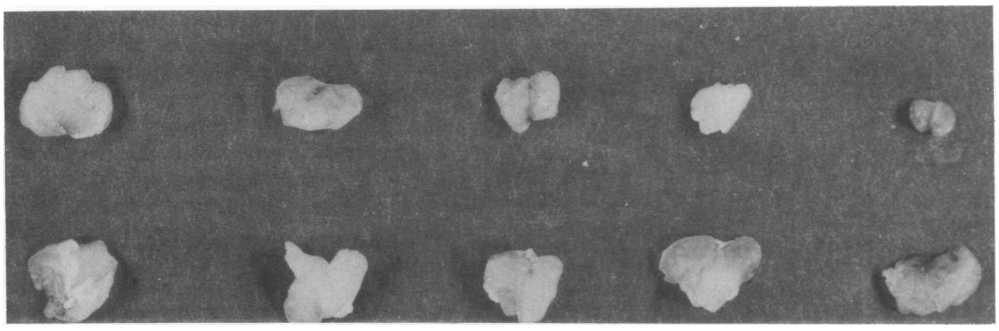

FIG. 4. *Comparison of the gross morphology of thymus from uninfected and from P3- and P9-infected mice. Top row (left to right): Thymus from uninfected mouse and thymuses from P9-infected mice 1, 3, 5, and 7 days after infection. Bottom row (left to right): Thymus from uninfected mouse and thymuses from P3-infected mice 1, 3, 5, and 7 days after infection.*

TABLE 4. *Number of cells obtained from thymuses of uninfected mice and at intervals after inoculation with 500 MID_{50} of P9 influenza virus*

Days after infection	Mean cell count[a] (× 10^6/ml)	Percentage of reduction[c]
0 (uninfected)	26.1 ± 5.3[b]	
3	16.0 ± 2.9	49
5	3.9 ± 1.9	85
7	0.43 ± 0.18	98

[a] Thymuses were removed and teased into individual single-cell suspensions, diluted, and counted in triplicate on a Coulter counter (Coulter Electronics, Hialeah, Fla., model ZBI).

[b] Mean value ± 1 standard deviation.

[c] Percentage of reduction in cells as compared with non-infected control values.

nude mice infected with P9 commonly revealed encephalomalacia and liquefaction (Fig. 8). Focal inflammation of the leptomeninges and mononuclear cell cuffing around penetrating vessels and vessels deeper within the cerebrum were common (Fig. 9). Inflammatory infiltrates consisting primarily of small lymphocytes were located extravascularly throughout the white matter. Ependymal degeneration and subependymal edema and perivascular cuffing were also noted. These histological findings are summarized in Table 5.

DISCUSSION

The increased lethality for mice acquired by a strain of Hong Kong influenza virus after six or more passages in these animals was not

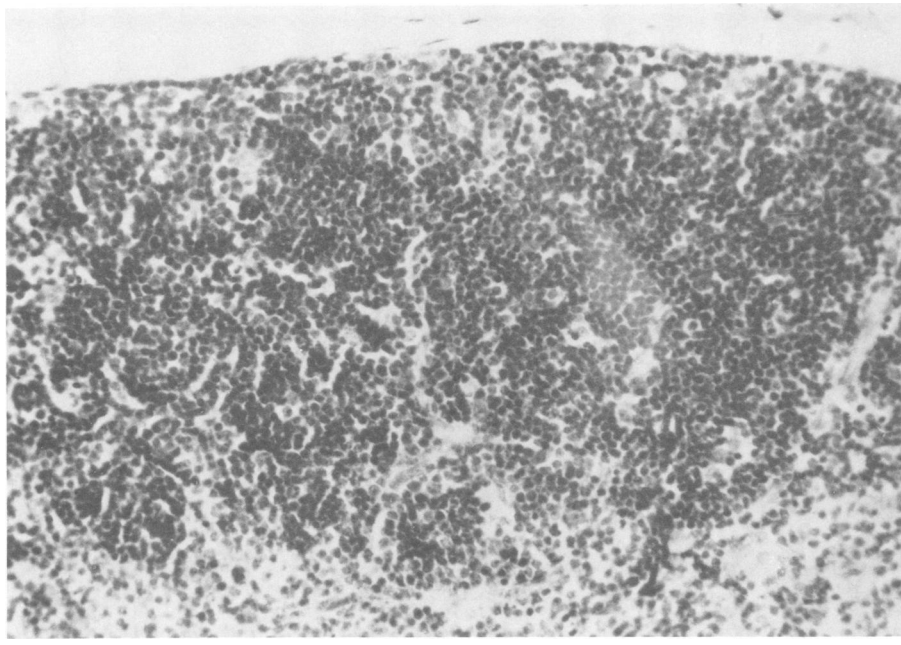

FIG. 5. *Photomicrograph of cortex of thymus from normal BALB/c mouse. Thymocytes fill the cortical areas. Hematoxylin and eosin stain (×100).*

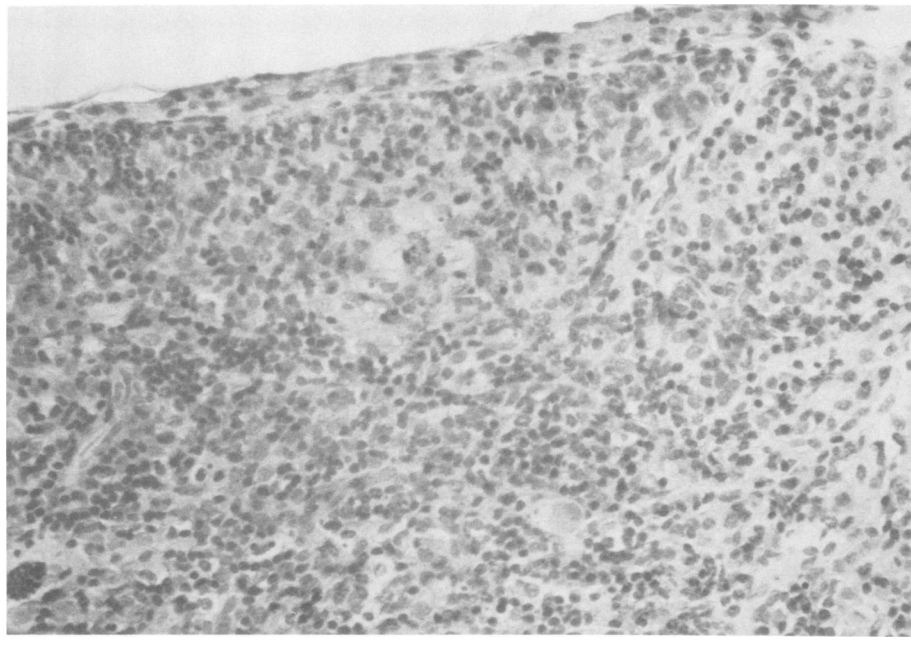

FIG. 6. *Cortex of thymus of P9-infected BALB/c mouse on day 5 illustrating the loss of cortical thymocytes. Hematoxylin and eosin stain (×100).*

associated in the present studies either with an increase in the titer of virus in the lung when compared to P2 through P5 or with dissemination of culturable virus to organs such as brain or spleen using infection with P9 virus as a model. The marked inflammation and destruction of lung tissue and the acute decrease in thymic size observed during the latter infection

contrasted with the minimal pneumonia and normal thymuses found during infection with P3 virus.

Influenza-induced destruction of mouse thymocytes (8) and marked atrophy of the thymus in humans with viral pneumonia have been reported (20, 21, 39). In the present studies, most of the decrease in the size of thymuses from mice infected with P9 virus appeared to be due to depletion of cortical lymphocytes. Although virus was recovered more frequently from thymuses of mice infected with P9 than with P3 virus, fluorescent-antibody localization of virus antigens in sections of thymuses revealed only occasional virus-positive cells in glands from the P9-infected animals. Signs of cellular destruction such as pyknotic cells, cellular debris, and polymorphonuclear cell infiltrates were minimal and appeared insufficient to account for the loss of cortical cells. The packing of regional lymphatics with small lymphocytes suggested instead that there may have been a large-scale emigration of thymocytes in association with the P9 virus infection.

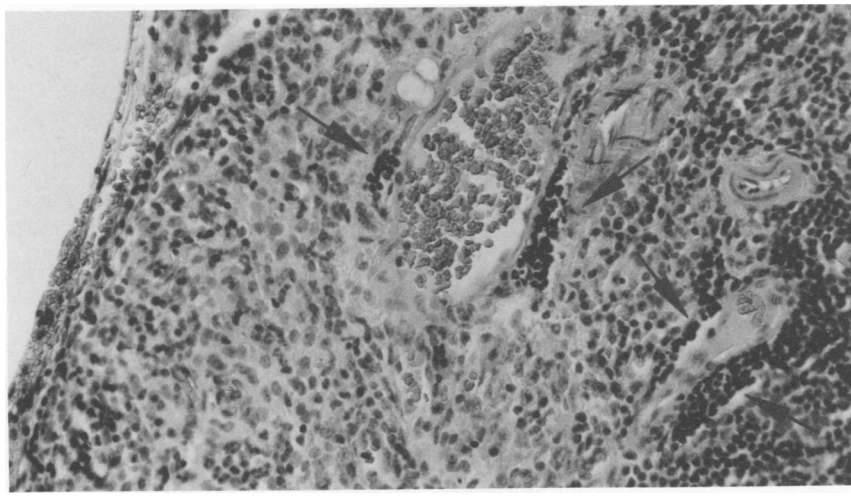

FIG. 7. *Photomicrograph of thymus from P9-infected mouse on day 5. The lymphatics are packed with small lymphocytes. Few lymphocytes are noted in the cortex. Hematoxylin and eosin stain (×50).*

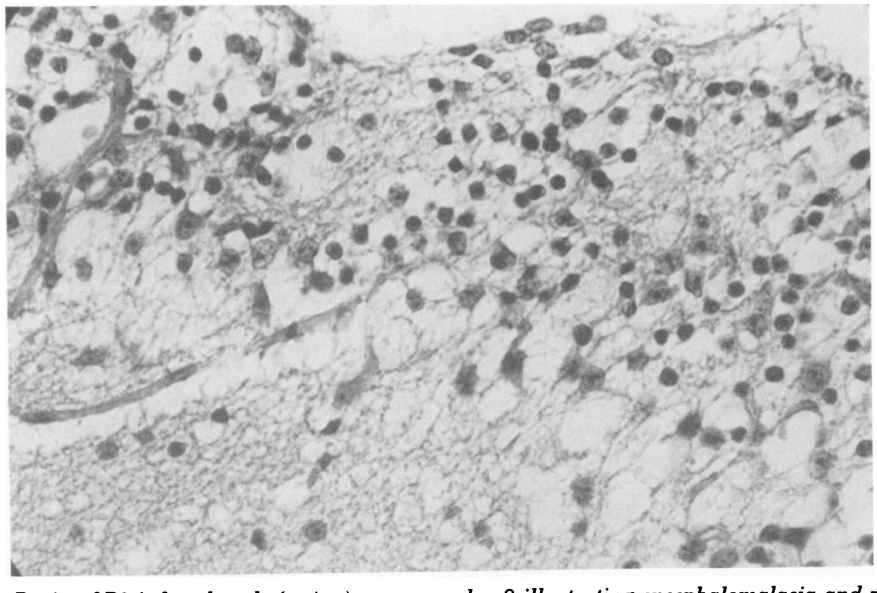

FIG. 8. *Brain of P9-infected nude (nu/nu) mouse on day 8 illustrating encephalomalacia and round cell infiltrate. Hematoxylin and eosin stain (×100).*

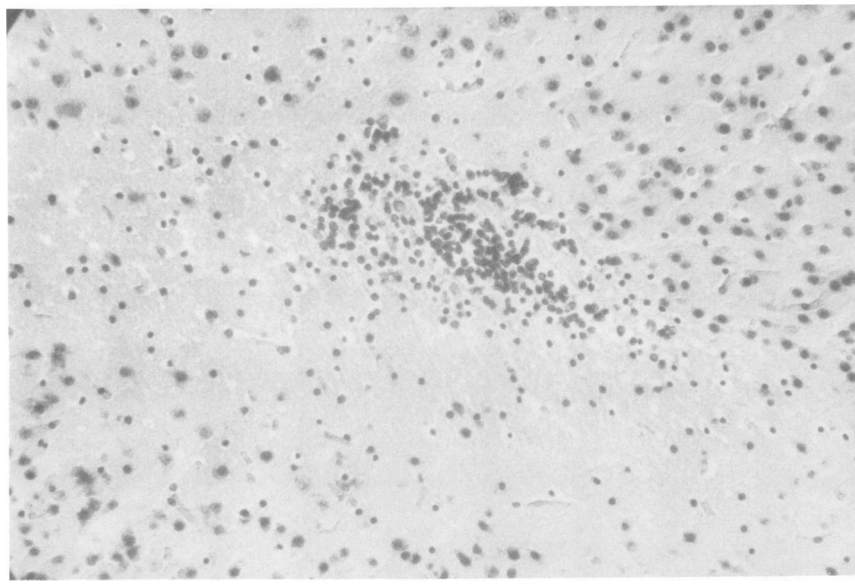

FIG. 9. *Photomicrograph of brain of 8-day high-passage-infected nude (nu/nu) mouse showing perivascular cuffing by small lymphocytes. Hematoxylin and eosin stain (×50).*

TABLE 5. *Histological findings in relation to passage level and immunocompetency*

Pathological finding	P3 virus (BALB/c mice)	P9 virus	
		BALB/c and furred Swiss mice	Nude Swiss mice
Lung			
Bronchitis	++[a]	++++	±
Bonchiolitis	++	++++	±
Pneumonia	±	++++	±
Thymus			
Cortical atrophy	0	++++	NA[b]
Brain			
Encephalomalacia, liquefaction, and perivascular cuffing	0	0	+++

[a] Plusses indicate relative degrees of manifestation of the finding, from not present (0) or trace (±) to extensive and severe (++++).

[b] NA, Not applicable.

Moreover, lymphocytes were prominent in the pulmonary inflammation that developed concomitantly with the decrease in thymic size. Further work will be necessary to determine whether and how these two time-related phenomena are interrelated mechanistically.

If thymus-dependent cells play a critical role in the inflammatory response to P9 influenza virus infection in the lung, then this inflammatory response should be considerably reduced in athymic nude mice. This was indeed the case since nude mice exhibited little inflammation or tissue disruption in their lungs during infection with this virus. Nude mice also had a more prolonged survival than immunocompetent animals after inoculation with P9 virus, but at the time of death they had higher titers of virus in their lungs, more frequent virus recoveries from brain, and histological evidence of encephalitis not seen in immunocompetent mice.

The results are compatible with the findings of others who studied the effects of antilymphocyte serum (33) and cyclophosphamide (30) on mouse influenza, and they suggest that the host's immune system plays a critical role in the inflammatory response to influenza virus in the lung. Potentially beneficial effects of this inflammation for the immunocompetent host, such as reduction in lung virus titers and prevention of virus dissemination to other organs, were obviated in the present studies by the overwhelming pneumonia induced by the dose and the virulence of the virus in the high-passage inocula. The data do not answer the question of how equivalent amounts of low-passage virus are localized and cleared from the lungs of immunocompetent mice with considerably less inflammation, but the contrasting models should prove useful for probing the host-virus interactions that characterize influenza virus virulence.

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