2001

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The FASEB Journal express article 10.1096/fj.01-0115fje. Published online June 8, 2001.

Host nutritional selenium status as a driving force for influenza virus mutations

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ABSTRACT

Previous work from our laboratory has demonstrated that infection with influenza A/Bangkok/1/79 (H3N2), a relatively mild strain of the virus, caused much more severe pneumonitis in selenium (Se)-deficient mice than in Se-adequate mice. Here we report that the increased virulence observed in the Se-deficient mice is due to mutations in the influenza virus genome, resulting in a more virulent genotype. Most of the mutations occurred in the gene for the M1 matrix protein, an internal protein that is thought to be relatively stable. A total of 29 nucleotide changes were observed in this gene, and all 29 changes were identical in three separate isolates taken from three different Se-deficient mice. In contrast, only one to three mutations were seen in the genes for the hemagglutinin or neuraminidase proteins, surface antigens that are known to be highly variable. Once the mutations have occurred, even hosts with normal nutritional status are susceptible to the newly virulent strain. This work, in conjunction with our earlier work with coxsackievirus, shows that specific nutritional deficiencies can have a profound impact on the genome of RNA viruses. Poor nutritional status in the host may contribute to the emergence of new viral strains.

Key words: selenium • influenza virus • oxidative stress • mutations • quasispecies

On a global basis, infections with influenza virus cause hundreds of thousands of deaths and millions of illnesses each year (1). In the United States alone, influenza kills more than 20,000 persons and hospitalizes more than 110,000 patients annually (2). Influenza virus readily undergoes mutation and genomic reassortment, thus making it difficult for the host immune system to recognize new strains. Previous work with coxsackieviruses demonstrated a rapid mutation to or selection of virulent genomes from avirulent genomes in selenium (Se)-deficient mice (3). Se is a nutritionally essential trace element required for the activity of a number of selenoenzymes, including the antioxidant enzyme, glutathione peroxidase (4). Mice deficient in Se developed myocarditis when infected with a normally benign strain of the coxsackievirus. This increase in virulence was due to six point mutations that occurred in virus

replicating in Se-deficient mice. Once the mutations had occurred, even mice of normal Se status were now susceptible to the increased virulence of the mutated virus.

The purpose of this study was to determine whether poor host Se status could similarly alter the genome of an influenza virus by increasing its rate of mutation. Our previous demonstrated that Se-deficient mice infected with influenza A/Bangkok/1/79 (H3N2) developed more severe lung pathology than infected Se-adequate mice did (5). Here we report that this increase in virulence was due to a change in the viral genotype.

MATERIALS AND METHODS

Mice

Three-week-old C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME) were housed 4/cage in the University of North Carolina animal facility and provided with food and water daily. Mice were fed specified diets for 4 wk before virus inoculation. All mice were maintained under protocols approved by the Institutional Animal Review Board of the University of North Carolina.

Diets

Diets were purchased from Harlan Teklad (Indianapolis, IN). Se was added to the adequate diets as sodium selenite. The Se level of the mouse diets was determined by continuous flow hydride generation atomic absorption spectrometry after acid digestion. The analysis was validated against NIST 1549 nonfat milk powder (National Institute of Standards and Technology, Gaithersburg, MD). The Se content of the experimental diets was determined to be 154 ± 8 μg Se/kg for the Se-adequate diet and below the instrumental detection limit of 2.7 μg Se/kg for the Se-deficient diet.

Virus

Influenza A/Bangkok/1/79 (H3N2) was propagated in 10-day-old embryonated hen's egg. The virus was collected in the allantoic fluid and titered by both hemagglutination (6) and 50% tissue culture infectious dose on Madin-Darby canine kidney cells (7). Stock virus was aliquoted in 0.5-mL volumes and stored at –80°C until needed.

Infection of mice

Mice were lightly anesthetized with an intraperitoneal injection of ketamine (0.022 mg) and xylazine (0.0156 mg). Following anesthesia, 10 hemagglutination units (HAUs) of influenza A/Bangkok/1/79 in 0.05 mL of phosphate-buffered saline were instilled intranasally, and the mice were allowed to recover from the anesthesia.

Histopathology of lungs

The right lung was removed, inflated with Optimal Cutting Temperature (OCT, Sigma, St. Louis, MO), diluted in phosphate-buffered saline, embedded in OCT, and immediately frozen on dry ice. Sections (6 μm) were cut on a cryostat and fixed and stained with hematoxylin-eosin. The extent of inflammation was graded without knowledge of the experimental variables by two independent investigators. Grading was performed semiquantitatively according to the relative degree (from lung to lung) of inflammatory infiltration. The scoring was as follows: 0, no inflammation; 1+, mild influx of inflammatory cells with cuffing around vessels; 2+, increased inflammation with approximately 25–50% of the total lung involved; 3+, severe inflammation involving 50–75% of the lung; and 4+, almost all lung tissue contains inflammatory infiltrates.

Determination of lung virus titers

One-quarter of the left lung (cut on the long-axis) was removed immediately after the mice were killed and frozen in liquid nitrogen. The lung tissue was weighed and ground, using a Tenbroeck tissue grinder, in a small volume of RPMI 1640 (Fisher Scientific, Pittsburgh, PA). Ground tissues were then centrifuged at 2000 × g for 15 min and the supernate recovered and titered by hemagglutination (6).

Viral passage

Mice were divided into two groups at weaning and fed either a Se-adequate or a Se-deficient diet (Harlan Teklad). Both groups of mice were infected with 10 HAUs of influenza A/Bangkok/1/79 (stock virus) intranasally following 4 weeks of consuming their respective diets. At day 5 postinfection, the mice were killed and one lobe of the lung was inflated and embedded in OCT. The remaining lobes were snap frozen in liquid nitrogen. Five randomly chosen lung samples from each diet group were weighed, ground in 150 μL of minimal essential media, and titered by hemagglutination. Ten HAUs of each recovered virus were used to inoculate five individual Se-adequate mice at 7 wk of age. Mice were killed at 6 days postinfection, and their lungs were examined for histopathology.

Viral sequencing

RNA was isolated from infected Madin-Darby canine kidney cells by freeze/thaw, centrifugation of the suspension, and addition of NaCl (final concentration 0.38 M) and 7% polyethylene glycol. Following an overnight incubation at 4°C, the cell lysate was centrifuged and the pellet resuspended in lysis buffer (0.5 M EDTA; 1.0 M Tris-HCl , pH 8.0; 5.0 M NaCl; 14.2 M β-mercaptoethanol; 10% SDS; 10mg/ml proteinase K) and incubated at 50°C for 1 h. Viral RNA was extracted from the samples with one phenol/chloroform extraction followed by one chloroform extraction. Glycogen, 10 M ammonium acetate, and ethanol were added, and the samples were kept at –80°C for 45 min. Samples were then centrifuged at 16,000 × g for 25 min, followed by a 75% ethanol wash of the pellet. The RNA pellet was air-dried for 15 min and stored at –80°C. cDNA was made by using random hexameric primers, following the Promega (Madison, WI) Reverse Transcription System manufacturer's instructions (Catalog # A3500). Primers were designed for amplification of the matrix (M), HA, and neuraminidase (NA) genes. PCR was performed with the following primer sets: M (accession number: K01140): 5'-GCACAGAGACTTGAAGATGT-3' and 3'-ATAGACTTTGGCACTCCTTC-5'; HA1

(accession number JO2092): 5'-CAGGTAGAATATGCGACAGT-3' and 3'-AGAACTGCAGGTGCCAATAG-5'; HA2: 5'-GATAACCGTGGACGTCAAGA-3' and 3'-TCCACAGGATCCAGTCTTTG-5'; NA1 (accession number KO1150): 5'-TTCAAGCAATATGAGTGCAGC-3' and 3'-CCTCTACATGCTGAGCACTTC-5'; NA2: 5'-CGTTCATATTAGCCCATTGTC-3' and 3'-ACTCTAGTTTCCTGTTCC-5'. PCR products were purified using the QIA quick PCR purification kit (Qiagen, Valencia, CA). DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a Model 377 DNA Sequencer (Applied Biosystems Division, Perkin Elmer, Boston, MA) by using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Applied Biosystems Division). HA gene was sequenced from nucleotide (nt) 181-810 and 855-1525 (total nt number for the HA gene is 1757). NA gene was sequenced from nt 152-823 and 827-1304 (total nt number for NA is 1392).

RESULTS

Se-adequate mice infected with virus recovered from Se-deficient mice develop severe lung pathology

Previous work in our laboratory (5) demonstrated that Se-deficient mice developed much more severe pathology postinfluenza virus infection than did Se-adequate mice, although viral titers were equivalent between the groups. To determine whether host factors or viral factors were responsible for the increased pathogenicity of influenza virus that had replicated in Se-deficient mice, a passage experiment was carried out.

Groups of weanling male mice were fed a diet either adequate or deficient in Se for 4 wk before infection. At day 5 postinfection (the time of peak lung pathology in both groups of mice), virus was isolated from the lungs of the Se-adequate mice and of the Se-deficient mice. Five separate viral isolates from five different Se-adequate mice and five separate isolates from five different Se-deficient mice were passed back into a second group of Se-adequate mice. If the increase in viral virulence were due to host factors alone, then the Se-adequate mice should not develop increased lung pathology when infected with virus recovered from Se-deficient mice as compared with Se-adequate mice infected with virus recovered from Se-adequate mice. However, we found that all five isolates from Se-deficient mice induced severe lung inflammation when inoculated into Se-adequate mice (mean score ±SD: 3.8±0.4), whereas all five isolates from Se-adequate mice induced only mild inflammation in the Se-adequate mice (mean score ±SD: 1.5±0.4), thereby indicating that a change had occurred in the virus itself (Fig. 1). The difference in lung pathology scores between the two groups was highly significant ($P<0.001$). There was no statistically significant difference in lung pathology scores between Se-adequate mice infected with the stock virus and Se-adequate mice infected with virus recovered from Se-adequate mice.

Sequencing of HA and NA regions of recovered virus

To determine whether a genome change had occurred in the virus that had replicated in the Se-deficient mice, we selected three viruses isolated from Se-adequate mice and three viruses isolated from Se-deficient mice for sequencing. Our stock virus of influenza A/Bangkok/1/79

(Flow Laboratories, McLean, VA) was propagated in the allantoic fluid of 10-day-old embryonated hen's eggs. Sequencing of our stock virus revealed that it was 99% homologous with previously published sequence data (GenBank accession numbers KO1140, JO2092, AF201843, AF008901, and AF008899, KO1150).

Sequencing of the HA gene of influenza virus allowed to replicate in Se-adequate or Se-deficient mice demonstrated one or two randomly distributed nt changes in viruses isolated from Se-adequate mice and two or three random nt changes in viruses isolated from Se-deficient mice compared with our stock virus (Table 1). One isolate from a Se-deficient animal had no nt changes compared with the stock virus. Two of the mutations were silent, and the remaining nt changes resulted in amino acid substitutions. At all other sequenced sites of the HA gene, the isolates were identical to the stock virus and to one another.

Sequencing of the NA gene revealed one nt change in each of two isolates from Se-adequate mice (Table 2). Both of these changes led to amino acid substitutions. The third isolate from a Se-adequate mouse had no changes compared with the stock virus. One isolate from a Se-deficient mouse contained two nt changes from the stock virus, each of which resulted in an amino acid substitution. The other isolate from a Se-deficient mouse did not differ from the stock virus.

Sequencing of M1 and M2 regions of recovered virus

In stark contrast to the HA and NA gene sequences, a large number of differences in the sequence of the M1 protein gene was found between viral isolates recovered from Se-deficient mice vs. those recovered from Se-adequate mice. As shown in Table 3, the sequence of the M1 protein gene determined from virus that had replicated in Se-deficient mice had 29 nt changes compared with the stock virus. Seven of these nt changes resulted in amino acid changes. All 29 nt changes were identical in all 3 isolates from Se-deficient mice. One isolate from a Se-deficient mouse had 5 more nt changes in addition to the 29 nt differences shared among all three isolates (Table 3). However, the sequence of the M1 protein gene of virus isolated from Se-adequate mice had no nt changes compared with the stock virus.

For the M2 protein gene, no changes were found in viruses recovered from Se-deficient mice. One isolate from a Se-adequate mouse had one nt change (nt 785) and this nucleotide change led to an amino acid change. The other two isolates were identical to the stock virus.

DISCUSSION

Previous work demonstrated that growing an amyocarditic coxsackievirus B3 in a host nutritionally deficient in Se resulted in several point mutations in the viral genome and conversion of the benign viral strain to virulence (3). Because Se is a component of the antioxidant enzyme glutathione peroxidase (GPX), it was suggested that increased oxidative stress in Se-deficient mice due to an absence of GPX activity was the driving force behind these viral changes. This idea received further support from the observation of Beck et al. (8) that a genetically induced lack of GPX in knockout mice resulted in similar genomic and virulence changes in the Picornavirus, coxsackievirus B3.

We wondered whether RNA viruses other than coxsackievirus were also susceptible to alterations in the host cellular redox environment. If the oxidative stress status of the host altered the genome of a virus outside the Picornavirus family, this would suggest that RNA viruses in general may be susceptible to oxidative damage. This would provide a novel mechanism for the emergence of viral diseases. To answer this question, we chose to examine the effect of host Se deficiency on influenza virus. Influenza A viruses are highly contagious and are responsible for considerable morbidity and mortality worldwide. New strains of influenza arise each year not only because of the propensity for influenza RNA segments to reassort but also because of the high mutation rate of genes that encode its surface antigens. We theorized that a decrease in host Se status might increase the mutation rate of the influenza virus.

Influenza viruses contain eight different single-stranded RNA segments that code for viral proteins. We chose to sequence three of the eight RNA segments that code for proteins that are associated with influenza virus virulence: the HA, the NA, and the M proteins (M1 and M2) (9, 10). The HA and NA are both located on the outer surface of the virus and are involved in entry into and budding from the host cell. The M1 protein (exclusively internal) and the M2 protein (an ion channel) are thought to increase virulence by accelerating the viral growth cycle. The M1 and M2 are coded for on the same RNA segment, with the M2 protein produced by alternative splicing of RNA segment.

The fact that the nucleotide changes were predominantly found in the M protein gene, rather than the HA or the NA genes, was an unexpected finding. The HA and the NA are exposed on the surface of the virion and are associated with antigenic changes of the virus. It was expected that any mutations found would be in the HA and/or the NA. The gene for the M1 protein is generally stable among influenza A viruses (11), which is thought to be due to M1 being an internal protein, and thus not subject to immune pressure from antibodies. The fact that the HA and NA were relatively stable in our study, whereas the M1 gene had a large number of changes, makes our findings all the more intriguing.

Our experiments did not involve the evolution of the influenza virus in multiple host mice over time, which is influenced by the immune pressure exerted by the host. Rather, our results reflect changes in the virus that occurred during its replication cycles in a single mouse. Thus, it seems likely that the heightened oxidative stress in the Se-deficient host during the viral replication cycle contributed to the increased mutation rate of the M gene.

Infection with influenza virus is known to increase oxidative stress in the host, and infection with influenza virus also up-regulates the expression of NF-κB, a redox-sensitive nuclear transcription factor (12–15). We hypothesize that the influenza viral infection, coupled with a lack of glutathione peroxidase activity due to host Se deficiency, led to a state of heightened oxidative stress in the Se-deficient mice. This increase in oxidative stress may have led to direct oxidative damage to the viral RNA, resulting in an altered genome with increased pathogenicity. It is significant that once these changes in the genome occurred, even mice with normal Se status were susceptible to the increased virulence of the mutated virus.

Recently, the existence of viral gene homologs for cellular glutathione peroxidase has been demonstrated in fowlpox virus and molluscum contagiosum virus (16, 17). The presence of a gene encoding for glutathione peroxidase in these poxviruses is postulated to help reduce the oxidative stress to which the viruses are exposed, clearly a survival advantage.

Another possible mechanism for the viral genome change in the Se-deficient mice is that of selection. The RNA-dependent polymerase of RNA viruses is highly error prone and lacking in proofreading capabilities. Therefore, within an individual host, an RNA virus exists as a heterogeneous mixture of closely related viruses called quasispecies (18). Within this mixture is the dominant, or consensus, sequence. The mutations that we found in the Se-deficient animals may have been generated by a selection process in which a new consensus sequence became dominant. The selection of the new consensus sequence may have been facilitated by the increased oxidative stress status of the host due to a deficiency in Se.

Our work points to the considerable influence that host nutritional status may exert on a viral pathogen. Together with the coxsackievirus model, our data with influenza virus suggest that many RNA viruses may be susceptible to nutritionally induced oxidative damage. We propose that the nutritional status of the host should be considered when exploring mutations and mutation rates of viruses. Finally, our results demonstrate a unique mechanism by which viruses can mutate and point to the importance of antioxidant protection against viral disease.

ACKNOWLEDGMENTS

This work was supported by a Nestec S.A. Supported Research Agreement (M.A.B.) and by a grant from the National Institutes of Health (DK56350) to the University of North Carolina Clinical Nutrition Research Unit.

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Received March 2, 2001; revised April 25, 2001.

Table 1

Comparison of nucleotide sequences of influenza A/Bangkok/1/79 gene for hemagglutinin (HA) protein in stock virus and in virus isolated from Se-adequate (Se+) or Se-deficient (Se-) mice[a].

	Virus Isolated From:							
Nt #[b]	Stock[c]	Se+[d]	Se+	Se+	Se-[e]	Se-	Se-	AA change[f]
435	C	C	C	C	A	C	C	None
456	C	T	C	C	T	C	C	None
494	A	A	G	A	A	A	A	N→S
500	C	C	C	T	C	C	C	T→I
579	A	A	A	A	A	C	A	K→N
583	T	A	T	T	T	T	T	I→V
637	A	A	A	A	A	G	A	Q→L
677	A	A	A	A	A	T	A	F→Y

[a] Nucleotides in boxes highlight changes from stock virus. HA gene was sequenced from nt 181-810, and 855-1525 (total nt number for the HA gene is 1757).
[b] Nucleotides read from 3'→5' direction.
[c] Stock virus was Influenza A/Bangkok/1/79.
[d] Influenza A/Bangkok/1/79 recovered from Se-adequate mice.
[e] Influenza A/Bangkok/1/79 recovered from Se-deficient mice.
[f] AA: Amino acid

Table 2

Comparison of nucleotide sequences of influenza A/Bangkok/1/79 gene for NA (neuraminidase) protein in stock virus and in virus isolated from Se-adequate (Se+) or Se-deficient (Se-) mice[a].

		Virus Isolated From:					
Nt #[b]	Stock[c]	Se+[d]	Se+	Se+	Se-[e]	Se-	AA change[f]
181	A	C	A	A	A	A	E→D
241	C	C	C	G	C	C	E→Q
925	A	A	A	A	G	A	V→I
1215	A	A	A	A	G	A	D→G

[a] Nucleotides in boxes highlight changes from stock virus. NA gene was sequenced from nt 152-823 and 827-1304 (total nt number for the NA gene is 1392).
[b] Nucleotides read from 3'→5' direction.
[c] Stock virus was Influenza A/Bangkok/1/79.
[d] Influenza A/Bangkok/1/79 recovered from Se-adequate mice.
[e] Influenza A/Bangkok/1/79 recovered from Se-deficient mice. Only 2 isolates were available for the sequence determination.
[f] AA: Amino acid

Table 3

Comparison of nucleotide sequences of influenza A/Bangkok/1/79 gene for M (matrix)1 and M2 protein in stock virus and in virus isolated from Se-adequate (Se+) or Se-deficient (Se-) mice[a].z

		Virus Isolated From:						
Nt #[b]	Stock[c]	Se+[d]	Se+	Se+	Se-[e]	Se-	Se-	AA change[f]
136	A	A	A	A	C	C	C	None
205	G	G	G	G	A	A	A	None
238	G	G	G	G	G	G	A	None
309	G	G	G	G	A	A	A	R→K(M1)
322	A	A	A	A	G	G	G	None
325	C	C	C	C	T	T	T	None
328	A	A	A	A	G	G	G	None
331	A	A	A	A	G	G	G	None
334	T	T	T	T	C	C	C	None
370	A	A	A	A	C	C	C	None
371	G	G	G	G	T	T	T	A→S(M1)
406	C	C	C	C	T	T	T	None
439	A	A	A	A	G	G	G	None
454	C	C	C	C	A	A	A	None
455	C	C	C	C	C	C	A	None
502	C	C	C	C	T	T	T	None
503	A	A	A	A	C	C	C	None
524	G	G	G	G	A	A	A	A→T(M1)
525	G	G	G	G	G	G	A	T→A(M1)
544	A	A	A	A	C	C	C	None
566	C	C	C	C	T	T	T	None
567	C	C	C	C	C	C	T	None
568	G	G	G	G	A	A	A	None
610	A	A	A	A	G	G	G	None
619	G	G	G	G	A	A	A	None
652	C	C	C	C	T	T	T	None
655	G	G	G	G	A	A	A	None
667	G	G	G	G	A	A	A	None
569	G	G	G	G	G	G	A	None
670	A	A	A	A	G	G	G	None
677	G	G	G	G	A	A	A	A→T(M1)
712	A	A	A	A	G	G	G	None
716	G	G	G	G	A	A	A	D→N(M1)
740	A	A	A	A	G	G	G	T→A(M1)
785	C	A	C	C	C	C	C	P→T(M2)

[a] Nucleotides in boxes highlight changes from stock virus. Matrix gene was sequenced from nt 119-952. (Total number of nts of matrix protein gene: 1028). M1 sequence is nt 26-784 and M2 sequence is 26-51 and 740-1007.
[b] Nucleotides read from 3'→5' direction.
[c] Stock virus was Influenza A/Bangkok/1/79.
[d] Influenza A/Bangkok/1/79 recovered from Se-adequate mice.
[e] Influenza A/Bangkok/1/79 recovered from Se-deficient mice.
[f] AA: Amino acid

Fig. 1

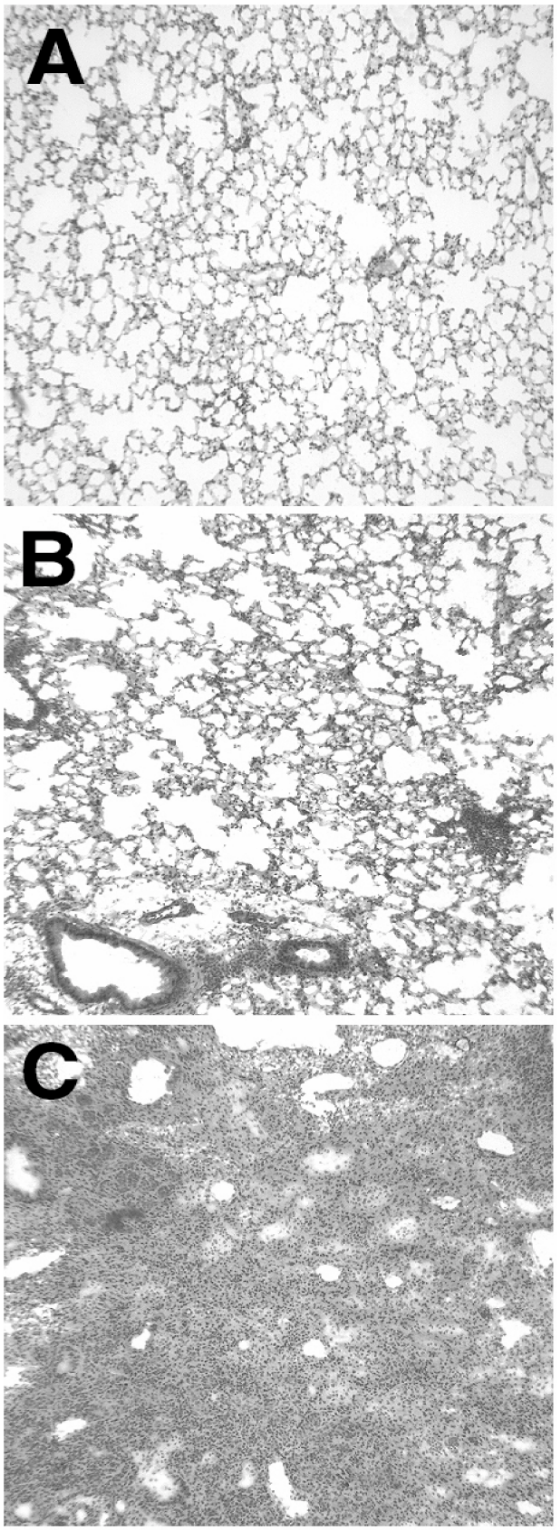

Figure 1. Representative lung histopathology of Se-adequate mice inoculated either with stock virus, with virus isolated from Se-adequate mice, or with virus isolated from Se-deficient mice. A) Lung section from Se-adequate mouse inoculated with 10 HAU stock influenza A/Bangkok/1/79 virus, **B)** lung section from Se-adequate mouse inoculated with 10 HAU influenza A/Bangkok/1/79 isolated from a Se-adequate mouse, and **C)** lung section from Se-adequate mouse inoculated with 10 HAU influenza A/Bangkok/1/79 isolated from a Se-deficient mouse. All sections were stained with hematoxylin and eosin. Magnification is ×40.