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Experimental influenza causes a non-permissive viral infection of brain, liver and muscle

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To determine whether some constitutional symptoms of influenza, such as headache, myalgia and nausea, could represent a viral infection of brain, muscle, and liver, we inoculated juvenile Balb/c mice intranasally with 10^3 plaque forming units of influenza B/Lee virus. Blood, brain, liver, skeletal muscle, and lung tissues were removed aseptically and assayed for infectivity by a plaque assay, viral RNA by reverse transcriptase-polymerase chain reaction (RT–PCR), viral antigen by immunoperoxidase staining, and histologic changes by light microscopy. Mice became ill 2–3 days post inoculation (PI). A productive viral infection of the lungs developed from days 1–8 with maxima of virus titers, pneumonia, and the number of immunoperoxidase staining lung cells occurring on days 2–6 PI. Virus isolation from blood was rare and viral RNA was detected intermittently in blood by RT–PCR. In many animals, a non-permissive or abortive infection of brain occurred from days 1–8 and peaked on days 3–4 PI. Viral RNA was detected in brain tissue and viral antigen was seen in cerebral endothelial cells but infectious virus was rarely isolated from brain. In liver, viral RNA was detected and viral antigen was seen occasionally in hepatocytes. In skeletal muscle, viral RNA was detected but neither infectious virus nor viral antigen was seen. A correlation existed between the severity of the illness, pneumonia, lung virus titer, viral antigen in lung cells, and extent of a non-permissive viral infection of brain and liver but not muscle. These studies demonstrate that following intranasal infection of influenza virus in mice, a viral pneumonia develops with subsequent intermittent viremia and non-permissive or abortive infection of brain, liver and muscle. *Journal of NeuroVirology* (2000) **6,** 529–536.

Keywords: influenza; influenza B virus; mice; polymerase chain reaction; non-permissive infection; abortive infection

Introduction

Human influenza viruses readily infect and replicate in cells lining the respiratory tract but seldom replicate in non-respiratory tissues. A minority of studies have isolated influenza virus from systemic organs (Engblom *et al*, 1983; LaMongagne, 1980; Partin *et al*, 1976). Even in overwhelming fatal influenza infections, influenza virus has not been commonly isolated from non-respiratory sites (Frankova *et al*, 1977; Kaji *et al*., 1959; Oseasohn *et al*, 1959). These observations have generally led to the proposition that human influenza seldom infects or replicates in non-respiratory cells (Mims, 1976). In contrast, certain avian influenza strains can replicate in cells of systemic organs of birds (Narayan, 1972).

In spite of these observations, human influenza B and A infections are characterized by prominent and frequent non-respiratory signs that often involve the neuromuscular system. Influenza B viral infection in children causes headaches in 60%, myalgias in 50%, and nausea and vomiting in 30% (Kilbourne, 1987). Furthermore, patients with influenza occasionally suffer more serious non-respiratory complications, such as myositis (Farrell

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et al, 1980; Gamboa *et al*., 1979), myocarditis (Engblom *et al*, 1983; Finland *et al*, 1945), vertigo (Gregson, 1949), deafness (Berg and Pallasch, 1981; Veltri *et al*, 1981) encephalopathy (Delorme and Middleton, 1981; Flewett and Hoult, 1958; Kimura *et al*, 1995; Protheroe and Mellor, 1991), and Reye's syndrome (Norman *et al*, 1968; Partin *et al*, 1976; Reynolds *et al*, 1972). Histopathological studies in patients dying with these non-respiratory complications, have implicated damage to brain (Frankova *et al*, 1977; Oseasohn *et al*, 1959), liver (Norman *et al*, 1968; Partin *et al*, 1976; Reynolds *et al*, 1972), skeletal muscle (Gamboa *et al*, 1979) and cardiac muscle (Finland *et al*, 1945). In general, the involved organ seldom contained infectious virus (Engblom *et al*, 1983; Farrell *et al*, 1980; Frankova *et al*, 1977; LaMontagne, 1980; Kaji *et al*, 1959; Kilbourne, 1987; Oseasohn *et al*, 1959; Reynolds *et al*, 1972) and the affected tissue often lacked inflammation (Farrell *et al*, 1980; Frankova *et al*, 1977; Norman *et al*, 1968). To explain the non-respiratory signs of influenza, some authors have suggested that the systemic organ damage was due to 'toxic' phenomena (Sweet and Smith, 1980) or to cytokines (Kurokawa *et al*, 1996; Price *et al*, 1997). To date a 'toxin' has not been clearly identified in influenza viral proteins. In mice and ferrets infected with human influenza A virus, elevated levels of tumor necrosis factor-α, interferon-γ, and interleukins 1, 2 and 6 have been found in pulmonary lavage fluid but not consistently in blood (Conn *et al*, 1995; Kurokawa *et al*, 1996; Price *et al*, 1997). In humans, experimentally infected with influenza virus, IL-6 has been recovered from nasal lavage fluid (Skoner *et al*, 1999).

Another possible explanation for the systemic signs is that they are not due to indirect or metabolic effects of influenza but due to direct viral infection of cells in systemic organs. However, if influenza virus does infect systemic tissues, the infection would have to yield little or no progeny virions and insufficient local viral antigens to elicit the typical inflammatory immune reaction that accompanies most viral infections. A non-permissive or abortive infection, i.e. one in which a virus enters a cell, partially replicates for a limited period of time, but without production of progeny infections virus could fulfil these criteria.

In earlier attempts to understand the pathogenic basis of brain and liver damage in Reye's syndrome, it was found that mice given high doses of influenza B virus intravenously (IV) developed a non-permissive viral infection of cerebral capillary endothelial cells and hepatocytes (Davis, 1987; Davis *et al*, 1983; Schwarz *et al*, 1991). These non-permissive organ infections reproduced many of the clinical, biochemical, and histological features of Reye's syndrome (Davis and Kornfeld, 1986).

In this study, we investigated whether influenza B virus inoculated intranasally (IN) in mice causes a similar, but milder, non-permissive viral infection of systemic organs.

Results

Clinical observations

Following IN inoculation of influenza B/Lee virus, most mice developed some ruffling of their fur on days 2–7 post inoculation (PI) and had decreased cage movement on days 3–7. Most animals displayed signs of greatest sickness on days 3–4 PI. After day 5, most animals became more active and resumed grooming. Minimal rhinitis was noted. Some animals never showed ruffled fur or illness behavior suggesting either they had a mild infection or were not infected.

Infectivity assays

Following IN inoculation, attempts were made to isolate virus from blood, brain, liver, lung and skeletal muscle on days 1–11 (Table 1). Virus was recovered from lung on all days in titers ranging from 10^3 to 10^6 PFU/gm. When virus was isolated from lung, the RT–PCR assay was also positive. Viremia was detected intermittently and only in low titers (4×10^2 to 5×10^3 PFU/ml). Virus was not isolated from liver or skeletal muscle. In the case of brain studies, virus was isolated once on day 3 in low titer (1×10^2 PFU/g) and confirmed by detection of viral RNA in the brain and viral antigen in cerebral capillary cells, from a mouse in which the blood culture was negative.

Viral PCR assays

Bands of 430 and 302 base pairs (bp) was seen on agarose gels when the influenza B outer and inner parts of matrix primers were used. The identity of the bands was confirmed by sequencing (data not shown). When influenza A primers were used on tissue from influenza B virus infected animals, no bands were seen. However, a single band of 943 bp was identified when the mouse G3PDH primers were used in both infected and non-infected mice. Table 1 shows the results, by organ, of detection of influenza B viral nucleic acid by RT–PCR on days 1–11 PI. Viral RNA was detected in blood intermittently during the first 8 days PI. In the brain, liver and muscle, viral RNA was detected daily for the first 7 days PI. PCR with influenza B primers was negative on tissues derived from A/PR8 infected mice (results not shown).

Whilst the question of sensitivity of detection of viral nucleic acid was not studied in a quantitative fashion, it was observed, for brain and liver samples, that detection was better from fresh tissue compared to ethanol-fixed, paraffin-embedded tissue sections. For lung tissue (which had high virus titers) no difference was noted.

Influenza B viral messenger RNA was detected using an Oligo $(dT)_{12-18}$ primer in the reverse

Table 1 Isolation of influenza virus and detection of influenza viral RNA by day after virus inoculation.

Post-inoculation day	*Lung #positive/#tested*	*Blood #positive/#tested*	*Brain #positive/#tested*	*Liver #positive/#tested*	*Skeletal muscle #positive/#tested*
1					
Virus isolation	3/3 (1.9×10^5)	0/6	0/2	0/6	0/6
Viral RT–PCR	7/7	3/7	2/7	1/6	1/6
2					
Virus isolation	3/3 (5.0×10^5)	0/3	0/2	0/6	0/5
Viral RT–PCR	3/6	0/6	2/6	1/6	2/6
3					
Virus isolation	3/3 (8.3×10^5)	0/4	1/4 (1.5×10^2)	0/5	0/4
Viral RT–PCR	6/6	0/6	5/5	3/6	2/6
4					
Virus isolation	3/3 (1.1×10^5)	3/7 (1.4×10^3)	0/9	0/10	0/4
Viral RT–PCR	11/11	2/10	10/11	7/11	4/11
5–7					
Virus isolation	4/7 (6.4×10^4)	0/10	0/8	0/15	0/0
Viral RT–PCR	12/15	5/15	7/15	6/15	3/15
8–11					
Virus isolation	2/2 (2.2×10^4)	0/3	0/2	0/4	0/1
Viral RT–PCR	9/12	1/7	2/7	0/8	0/7

Numbers in parenthesis () represent the mean infectious titer in PFU/g of tissue or PFU/ml of blood. Each sample in each tissue category was from a different mouse.

transcriptase step followed by PCR using specific primers in 26/31 ethanol or formalin fixed lung, brain and liver tissues on days 1, 2 and 3 PI. Fresh tissue was not assayed for mRNA.

Histology and immunohistochemistry

On days 1 and 2 PI, the lungs appeared well aerated, pink and of normal appearance. Histologic sections showed large areas of normal architecture and tiny foci of infiltration of interstitial tissue with macrophages or lymphocytes. Few neutrophils were seen. Immunoperoxidase (PAP) staining demonstrated occasional positive large mononuclear cells suggestive of macrophages within alveoli. Smaller alveolar cells were rarely positive. Bronchi and bronchioles showed scattered positive endothelial cells. On days 3–6 PI, some lungs appeared almost normal while others had larger foci of edema. The number of mononuclear inflammatory cells within alveoli varied from rarely present to markedly increased. The foci of inflammatory cells were spotty but were never heavy. PAP positive cells varied from occasional to fairly heavy in some areas. PAP positive cells were seen in the alveolar lining and in bronchi and bronchioli (Figure 1A). The lungs of two mice on day 4 had areas of bronchopneumonia and the presence of neutrophils. There was a correlation between the number of PAP positive cells in the lung and the infectious titer in the lung. When the mouse blood was PCR positive for viral RNA, the animal's lungs were always congested but not all animals with congested lung or infectious virus therein had PCR positive blood. After day 7, resolution of the lung infection began. The mean lung infectious titer fell 10-fold and the lungs appeared near normal. A few foci of resolving consolidation were present and foci of strongly positive PAP staining alveolar cells could still be seen in some animals. While most of these mice appeared to have cleared the respiratory infection, it is possible that some mice were never infected. In mice with histologically near normal lungs, no animals had blood that was PCR positive for viral RNA. In contrast, nine of 20 mice with lung congestion had PCR positive blood.

The brains, fixed in ethanol and stained with H&E appeared histologically normal on all days PI. On days 1–7, occasional cerebral capillary endothelial cells were PAP positive (Figure 1B). There was a correlation between the detection of influenza viral RNA by RT–PCR assay on brain and the presence of immunoperoxidase positive endothelial cells. On days 8–11, the number of endothelial cells that stained positive decreased but foci of fading positive cells could still be seen.

In the liver, histologic abnormalities were seldom seen by light microscopy. In three livers on days 2, 3 and 4, the cytoplasm of hepatocytes was vacuolated as seen by H&E. In these livers, the same liver areas had PAP positive staining of hepatocyte nuclei (Figure 1C).

In the quadriceps muscle, no histological abnormalities were seen. PAP staining was not performed.

Clinical-virologic-pathologic correlations

There was a correlation between the severity of clinical illness, the extent of the pneumonia, and the per cent of animals with viral RNA detected in blood brain and liver (i.e. showing viremia). Over

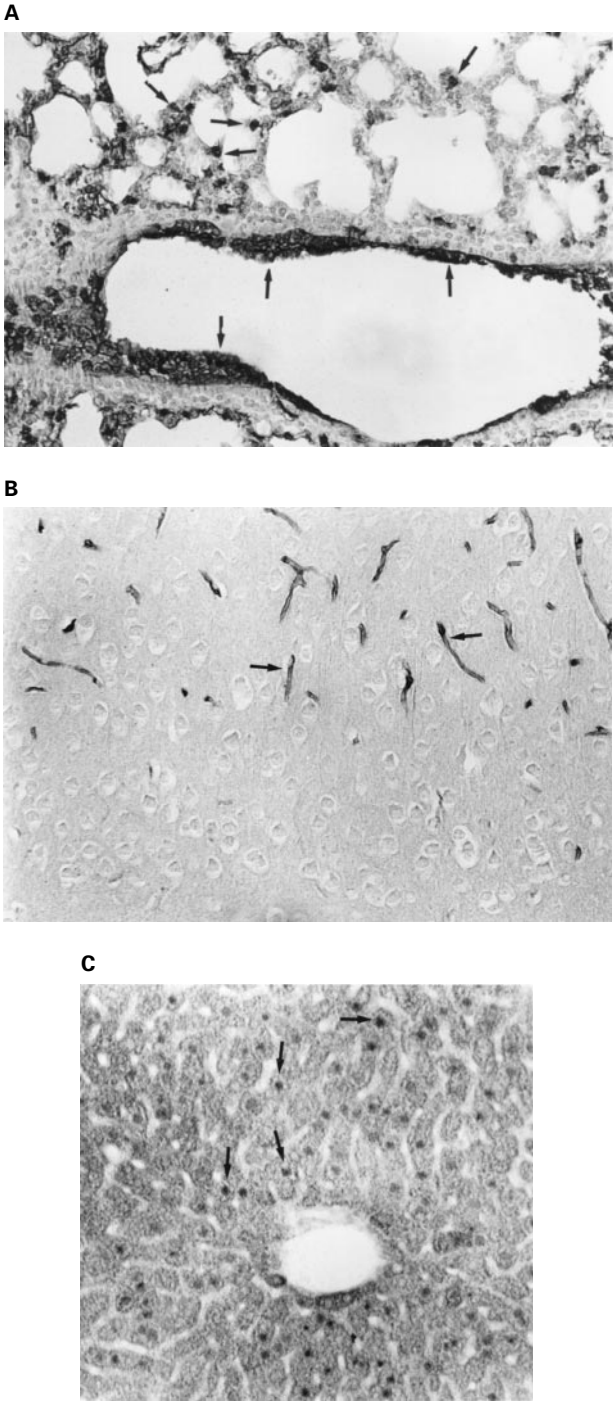

Figure 1 Immunoperoxidase staining with rabbit anti-influenza B/Lee antiserum of 6-μm thick sections of ethanol fixed and paraffin embedded organs from Balb/c mice after intranasal inoculation of influenza B/Lee virus. (**A**) Lung 5 days PI, viral antigen in large alveolar cells (likely macrophages) (arrows) and bronchiolar epithelia cells (arrows). (**B**) Cerebral cortex 2 days PI, with viral antigen seen within scattered endothelial cells (arrows). No other parenchymal cells demonstrated specific staining. (**C**) Liver section 3 days PI, demonstrating scattered hepatocytes with specific staining of nuclei (arrows). Narmoski optics ($\times 200$) were used.

the period of observation, 25 mice showed only mild illness behavior or appeared normal while 27 mice demonstrated moderate to marked illness bahavior. Five mice showed no illness behavior. The ill mice had a mean lung infleunza virus titer of 3.1×10^5 PFU/gm (median of 1.3×10^5 and range of 1.1×10^3 to 1.4×10^6) compared to a mean lung titer of 1.0×10^3 PFU/gm (median of 4×10^3 and range of negative to 3.2×10^5) for well mice.

The lungs of ill mice showed moderate to severe congestion with 2 to 3+ positive alveolar cells by immunoperoxidase staining in 84% and mild congestion with 1+ positive cells in 16%. In well appearing mice, the reverse occurred and only 16% of lungs showed moderate to severe congestion with 2 to 3+ specific cell staining, whilst the remaining 84% showed no or only mild congestion and negative or 1+ specific cell staining. Seventy-five per cent of brains from ill mice contained viral RNA compared to 33% from well mice. This approximate 2 : 1 ratio for viral RNA ill : well mice was seen in liver (50% : 26%) and blood (28% : 8%) but not in muscle (28% : 26%). Ill mice were the source of three virus isolations from blood.

Discussion

This study represents an important extension of earlier work. In previous studies, we and others reported that IV inoculation of a high dose ($\geqslant 3 \times 10^6$ PFU) of influenza B/Lee into juvenile Balb/c mice caused a widespread, non-permissive infection of cerebral endothelial and hepatocytes (Davis, 1987; Davis *et al*, 1983, 1990; Schwarz *et al*, 1991). In this high dose IV model, mice developed seizures, coma and died 1–4 days PI. In the brain, perivascular edema developed and most cerebral capillary cells contained viral antigen. The liver demonstrated widespread fatty degeneration and many hepatocytes contained viral antigen. The brain and liver infections were non-permissive. This proved to be a reasonable model of Reye's syndrome but not for influenza as severe brain and liver disease are not seen in infleunza.

When mice were inoculated IV with a lower dose of influenza B virus ($< 1 \times 10^6$ PFU), the mice appeared mildly ill and did not develop seizures or coma. Their brains and livers were histologically normal, but specific immunofluorescent staining of brain endothelial cells and hepatocytes was seen (Davis and Wallace, 1995). Again, the brain and liver infections were non-permissive. While this model suggested that influenza infection may produce similar findings, the IV inoculation route was a non-natural route of infection. To produce an experimental model closer to clinical influenza, we inoculated mice intranasally with the same virus.

The experiments reported here demonstrate that influenza B virus administered intranasally resulted

in a productive infection of cells of the lower respiratory tract and a non-permissive infection of other systemic organs. The pneumonia was maximal on days 2–4 as evidenced by the intensity of the animal's illness, extent of the pneumonia seen histologically and period of highest lung viral titers. The character and time course of our viral pneumonia was similar to a previous report (Loosli, 1949). There was variation in the severity of the viral pneumonia between animals likely due to the difficulty in delivering the same viral dose to the lower respiratory tract by the intranasal route.

In some mice we documented a viremia and in more animals we detected viral RNA in blood. The magnitude of the viremia was always low and approached the limits of our viral isolation method. Thus, it is possible the viral RNA detection represented viremias of less than 100 PFU/ml blood. Our findings are similar to other studies of IN influenza A virus inoculation of mice that reported intermittent virus isolation from blood (Hamre *et al*, 1956) or virus RNA detection RT–PCR (Mori *et al*, 1995). All these studies suggest the viremia is transient and of low titer. Previous explanations for the brief low titered viremia have included intermittent release of virus into blood from the respiratory tract infection, rapid removal of virus from blood by the reticulo-endothelial system, and non-specific inhibitors in normal serum. To this list we add attachment and penetration of influenza virus to cerebral and possibly other systemic organ endothelial cells resulting in a non-permissive infection with failure of production of progeny virions. This contrasts with certain strains of avian influenza virus which can fully replicate in vascular endothelial cells and produce a high titered viremia (Kobayashi *et al*, 1996).

The transient viremia in our mice appeared insufficient to infect cerebral endothelial cells but the infection did not produce cerebral edema. Evidence for the cerebral capillary cell infection being non-permissive comes from identification of specific viral antigen within endothelial cells but no spread of staining to adjacent neurons or glia and the inability to isolate virus from the brain. In addition, there was a uniform fading of the endothelium staining intensity suggesting that new cycles of infection did not occur even in the endothelial cells. The possibility of influenza virus reaching the brain via a neural route was considered as a neural route of spread from infected respiratory tract cells to the brain via cranial nerves appears to occur in mice infected IN with the recently isolated Hong Kong (H5N1) influenza 156/97 and 483/97 virus (Gao *et al*, 1999). However, in H5N1 infected mice, viral antigen was found within neurons at specific brain locations and not in endothelium. In our model, viral antigen has only been identified within cerebral endothelial cells, suggesting that a blood-borne route is more likely.

The transient viremia also infected occasional hepatocytes and muscle cells as evidenced by specific staining of an occasional hepatocytes and detection of viral RNA in both organs. Our findings are consistent with an experimental murine study of influenza A virus in which viral RNA and mRNA to the nucleoprotein gene were demonstrated in liver, skeletal muscle and brain following IN inoculation (Mori *et al*, 1995).

Although we demonstrated a correlation between the severity of the clinical illness and the frequency of viral RNA in blood, brain and liver, we found that even mildly ill mice could develop non-permissive viral infections of systemic organs.

A question arises as to why we did not identify influenza virus more often in systemic organs by the RT–PCR assay. One possibility is that virus escapes the lung to infect systemic organs in relatively few animals. Another possibility is that non-respiratory host cells do not support complete replication of influenza resulting in non-permissive or abortive infection. Such infections may produce limited amounts of viral RNA for a brief period of time, thereby rendering success of the RT–PCR procedure somewhat sporadic. It has been shown, by immunofluorescent antibody studies of infected tissue culture cells (Cole, 1983) and previous murine studies (Davis, 1987), that the period of active influenza infection is brief. Viral proteins are detected maximally at 12–24 h PI with loss of detectable viral proteins by day 3 (Davis, 1987). The resulting non-permissive infection is sufficiently brief that the infection seldom kills the endothelial cell or hepatocyte (Davis *et al*, 1983; Davis, 1987). While we have not performed RT–PCR assays that are semi-quantitative, we found that in permissively infected cells, the PCR assay is positive using only the outer primers while for non-permissively infected cells nested RT–PCR assay is often necessary to detect viral RNA. This suggests that the amount of viral RNA produced within an abortively infected cell is considerably less than in cells that allow complete viral replication. As a consequence, it is possible that our infected mice developed sufficiently brief non-permissive infections of systemic organs that the organ did not always contain detectable viral RNA.

There is some evidence that the non-respiratory symptoms common in human influenza infection (headache, nausea, and myalgia) could be caused by non-permissive viral infection of brain, liver, and muscle. Viremia following uncomplicated human influenza has been documented (Khakpour *et al*, 1969; Lehmann and Gust, 1971; Naficy, 1963; Stanley and Jackson, 1966; Xu *et al*, 1998). Patients with influenza may have elevated serum levels of creatinine phosphokinase (Friman, 1976) and liver transaminases (Lichtenstein *et al*, 1983; Louria *et al*, 1959) suggested mild damage to muscle and liver. Central nervous system involvement in influenza

has been suggested by Fujimoto *et al* (1998) who identified influenza viral RNA, by RT–PCR, in the CSF of five patients.

Materials and methods

Virus

Influenza B/Lee/40 virus was grown in the allantoic cavity of 10 day embryonated chicken eggs (Davis, 1987; Davis *et al*, 1983). Virus pools were concentrated by ultracentrifugation and had an infectivity titer of 10^7 to 10^8/ml plaque forming units (PFU) in Maden Darby canine kidney (MDGK) cells (Tobita *et al*, 1975). Virus pools were stored at -70°C until diluted in phosphate buffered saline (PBS) and used.

Animals

Fifty-seven Balb/c mice, age 2–4 weeks, were lightly anesthetized by intraperitoneal administration of 0.3 mg/kg of chloral hydrate and inoculated with 10^3 PFU virus intranasally. Mice were observed daily for behavioral changes and scored as well (active, normally groomed, and feeding), mildly ill (less active with partial grooming) and very ill (little movement about the cage and failure to groom or eat). All but three animals, sacrificed as they showed signs of severe illness, were randomly selected and killed 1–11 days PI by administering an overdose of chloral hydrate followed by exsanguination and cardiac perfusion with sterile PBS. Blood and the following organs were removed aseptically; brain, liver, lung, and skeletal muscle. Each organ was removed with individual sterilized instruments and kept separately. Tissue pieces were immediately ground into a 10% homogenate with PBS or fixed in 70% ethanol followed by paraffin embedding. Tissues for 20 mice were also fixed in 10% formalin followed by paraffin embedding. Tissues fixed in ethanol or formalin were used in the PCR assay done at the UK laboratory and fresh tissues were used by the Albuquerque PCR laboratory. Thus, all tissues were not processed by all methods and for technical reasons, all assays were not performed for every animal.

Infectivity assay

Fresh tissue was homogenized into a 10% suspension and inoculated onto small French square bottles containing MDCK cells for a plaque assay (Tobita *et al*, 1975). The lower limit of the plaque assay was 50–100 PFU/ml.

Immunohistochemistry

Tissues fixed in ethanol and paraffin embedded (Davis, 1987; Davis *et al*, 1983) were cut into 6-μm thick sections and deparaffinized. Primary antisera used were normal rabbit or goat sera, commercial goat antiserum to group B nucleoprotein and matrix antigens (Chemicon International Inc.), and rabbit sera raised by immunization with egg-grown influenza virus, either B/Lee or A/PR8, which had been absorbed with guinea pig red blood cells (Davis, 1987; Davis *et al*, 1983). Commercial peroxidase or fluorescein labeled anti-rabbit or anti-goat antibody was used as the secondary reagent. Indirect immunoperoxidase and immunofluorescence techniques were performed as previously reported (Davis, 1987; Davis *et al*, 1983). A semi-quantitative scoring system for the per cent of lung alveolar cells in the lung section that showed specific staining was as follows: 1+=<10%, 2+=10 to 29%, 3+=30 to 50%, and 4+=>50%. Adjacent sections usually were stained with hematoxylin and eosin (H&E) for histologic examination.

Polymerase chain reaction studies of influenza viral RNA

Extracts of fresh tissue were used in the RT–PCR assays performed in the US laboratory. Adjacent tissue was fixed in ethanol or formalin and paraffin embedded. The RT–PCR assay was carried out on extracts of these fixed samples in the UK laboratory. RNA was extracted from fresh tissue and whole blood (0.1 ml placed directly in lysis buffer) using the method and reagents of the Ultraspec™ II RNA purification kit (Biotecx Labs) and precipitated overnight with ethanol at -70°C. RNA was extracted from ethanol or formalin fixed, paraffin embedded blocks by cutting 6–10 sections, each 10 μm thick, and digesting them with 1 mg/ml proteinase K at 37°C for 3 days followed by standard RNA extraction (Jackson *et al*, 1990).

Nested primers designed to hybridize to conserved regions of the influenza B virus matrix gene were used (Zhang and Evans, 1991). Outer primers (BMPA-TGTCGCTGTTTGGAGACACA [position 26–45]; BMPDII-AGTTTTACTTGCATTGAATA [position 455–436]) defined a 430 nucleotide sequence from 5′–26 to 455 and were used to prime cDNA synthesis at 0.5 ng/μl. Inner primers (BMPB-GAAGGCAAAGCAGAACTAGC [position 79–98]; BMPGII-TGGCCTTCTGCTATTTCAAA [position 380–361]) defined a 302 nucleotide sequence from 5′–79 to 380 and primed PCR reactions at 1.25 ng/μl. Control primers included a 943 nucleotide sequence in the murine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene (Clontech) and a nested set of influenza A virus matrix gene primers yielding products of 640 (AMPA–CCGTCAGGCCCCCTCAAAGC [position 71–90]; AMPDII-GACCAGCACTGGAGCTAGGA [position 710–691]) and 401 (AmPB–CAGAGACTTGAAGATGTCTT [position 101–120]; AMPCII–TGCTGGGAGTCAGCAATCTG [position 501–482]) nucleotides.

To convert RNA to cDNA, random hexamers (3 μg/ml; Gibco BRL) or one outer influenza B virus primer (BMPA) or Oligo $(dT)_{12-18}$ primers were used with M-MuLV reverse transcriptase (Perkin Elmer

RNA PCR kit). The reaction was performed at 30°C for 10 min followed by 42°C for 60 min. As a control, the outer influenza A virus matrix gene primer, AMPA, was used.

In the PCR step, amplification of the cDNA was performed with either the Perkin Elmer RNA-PCR kit in a Perkin Elmer 480 thermal cycler or Stratagene Robocycler for 35 cycles at annealing temperatures between 35–45°C depending on the primer and extension at 72°C (Zhang and Evans, 1991). Control primers included both outer influenza A matrix gene primers and both mouse G3PDH gene primers (Clontech). On completion of cycling, the tubes were cooled to 4°C to solidify the PCR GEM. Both rounds of the nested PCRs were visualized on 2% agarose gels in TBE buffer (0.45 M Tris-borate 0.001 M EDTA) with 0.5 μg/ml ethidium bromide.

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