Characterization of human influenza A (H5N1) virus infection in mice: neuro-, pneumo- and adipotropic infection

Hidekazu Nishimura,1 Shigeyuki Itamura,1 Takuya Iwasaki,2 Takeshi Kurata2 and Masato Tashiro3

Department of Virology I1, Department of Pathology2 and Department of Viral Diseases and Vaccine Control3, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan

Mice (ddY strain, 4 weeks old) were infected intranasally with the H5N1 influenza viruses A/Hong Kong/156/97 (HK156) and A/Hong Kong/483/97 (HK483) isolated from humans. HK156 and HK483 required 200 and 5 p.f.u. of virus, respectively, to give a 50% lethal dose to the mice when the volume of inoculum was set at 10 µl. Both viruses caused encephalitis and severe bronchopneumonia in infected mice. The severity of lung lesions caused by the viruses was essentially similar, whereas HK483 caused more extensive lesions in the brain than did HK156. This was supported by the results of virus titration of organ homogenates, which showed that the virus titres in brains of HK483-infected mice were more than 100-fold higher than those of HK156-infected mice, while those in lungs were almost equivalent. Both viruses were detected in homogenates of the heart, liver, spleen and kidney and blood of the infected mice. Virus antigen was detected by immunohistology in the heart and liver, albeit sporadically, but caused no degenerative change in these organs. The antigen was not detected in the thymus, spleen, pancreas, kidney or gastrointestinal tract. In contrast, virus antigen was found frequently in adipose tissues attached to those organs. The adipose tissues showed severe degenerative change and the virus titres in the tissues were high and comparable to those in lungs. Thus, infection of HK156 and HK483 in our mouse model was pneumo-, neuro- and adipotropic, but not pantropic. Furthermore, HK483 showed higher neurotropism than HK156, which may account for its higher lethality.

Introduction

An outbreak of influenza A (H5N1) virus infections occurred in birds in poultry farms and markets and in residents in Hong Kong in 1997. Human cases, which were detected for the first time, eventually numbered 18.

All of the human isolates of the H5N1 virus possessed haemagglutinin (HA) and neuraminidase genes of avian origin and all of the other genes of one of the human isolates, A/Hong Kong/156/97 (HK156), were characterized as of avian origin (Subbarao et al., 1998; Claas et al., 1998; Suarez et al., 1998), and the isolated H5N1 viruses were divisible into two groups, represented by HK156 and A/Hong Kong/483/97 (HK483), according to the antigenic and phylogenetic properties of HA (Suarez et al., 1998; Bender et al., 1999).

Avian influenza viruses had not been thought to be pathogenic in humans, except for reports of mild cases of conjunctivitis caused by the H7N7 subtype (Webster et al., 1981; Kurtz et al., 1996), although they have caused fatal outbreaks of influenza several times in wild and domestic mammalian species (Hinshaw, 1998). Clinical features of the 18 human cases in this outbreak of H5N1 influenza varied from mild illness to severe illness, including six cases of death (Yuen et al., 1998), although, epidemiologically, the number of non-apparent cases in the community during the outbreak was not available. Both the apparently high case-fatality rate among the confirmed cases and the novel antigenicity of the emerged viruses for humans suggested a great threat of a large-scale outbreak of highly virulent influenza in humans. In response to this, trials to develop vaccines against H5N1 influenza virus were commenced at several places worldwide, including our laboratory. We employed the technique of reverse genetics and made a recombinant virus as a candidate strain for vaccine manufacture: we modified the HA gene of HK156 so that its deduced HA molecule lacked the basic amino acid array at its
protease-cleavage site and introduced the gene into an avirulent, avian influenza A (H3N1) virus (unpublished results).

In assessing safety issues and evaluating the potency of the recombinant as the candidate vaccine strain, we needed some proper animal model with which infection experiments with the recombinant and wild strains and immunization experiments with the candidate vaccine could be performed. HK156 and HK483 caused highly lethal, systemic infection in chickens and pathological features in chickens infected with HK156 have been well studied (Suarez et al., 1998). Mice have proved to be a good mammalian model for human H1N1, H2N2 and H3N2 influenza, since the pathology of the lungs of infected mice was similar to that of severe cases of influenza in humans. Mice were therefore used for the H5N1 influenza viruses as well, and it was revealed that some human isolates of H5N1 influenza virus were highly virulent to mice without any adaptation step. Consequently, the mouse model was used in challenge and protection experiments with the H5N1 viruses and candidate vaccines (Li et al., 1999; Lu et al., 1999; Takada et al., 1999). In the H5N1 virus–mouse models, live virus and/or viral antigen were recovered in some organs of infected mice, suggesting that the infection was pantropic (Gubareva et al., 1998; Shortridge et al., 1998; Gao et al., 1999; Lu et al., 1999).

Histopathological analyses of organs are essential in discussing the pathogenicity of infectious agents in vivo, especially when the possibility of pantropic infection is suspected. It has been reported that HK483 caused pneumonia, encephalitis and degeneration of cardiomyofibres (Gao et al., 1999; Lu et al., 1999) and that HK156 caused severe respiratory tract lesions (Dybing et al., 2000) in BALB/c mice. Nevertheless, information on other organs and from a viewpoint of comparison of pathological features among virus strains, based on histopathological analysis, is not fully available to date.

In this study, we performed infection experiments in mice with two H5N1 influenza viruses, HK156 and HK483, which were isolated from deceased patients in the outbreak and represent the two distinguishable antigenic/phylogenetic groups among the isolated H5N1 viruses, and attempted the detailed pathological and virological investigation of infected mice.

Methods

- **Viruses and mice.** All experiments using live viruses were conducted in biosafety level 3 facilities. All animal experiments were conducted in conditions based on the Guiding Principles on Animal Experimentation of the National Institute of Infectious Diseases, Japan. Four-week-old, specific-pathogen-free, out-bred white mice, ddY strain (SLC Co., Hamamatsu, Japan), were used throughout this study. The H5N1 viruses HK156 and HK483 were isolated with Mardin–Darby canine kidney cells (MDCK) at the Government Virus Unit, Queen Mary Hospital, Hong Kong. The seed viruses used in experiments were obtained by propagation in MDCK cells in the presence of 10 µg/ml acetylated trypsin (Sigma), without a history of passage in mice. HK483 was passed three or four times in MDCK cells and HK156 was passed once in the allantoic cavity of embryonated hen’s egg and twice in MDCK cells before the final propagation step for seed preparation was performed in MDCK cells. Egg-grown A/PR/8/34 (H1N1) virus seed was kindly provided from Dr Tamura of the Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan.

- **Infection and virus titration.** For infection, mice were anaesthetized with pentobarbital and the virus suspension was dropped into the nostrils. The volume and infectivity titre of the inoculum were set at 20 µl (10 µl per nostril) and 10<sup>5</sup> p.f.u./mdCK in MDCK cells throughout this study unless mentioned otherwise. For virus titration of organs and blood of infected mice, each organ was excised and washed three times in PBS pH 7.2. Blood was drawn and left at room temperature for more than 30 min. Organs and blood were kept at –80 °C without thawing until the titration was commenced. Each organ or blood sample (clot and serum) was ground with PBS to make a 10% (v/v) homogenate. The homogenate was cleared by centrifugation at 3000 r.p.m. for 20 min and the supernatant was subjected to plaque assay using MDCK cells in the presence of 10 µg/ml acetylated trypsin.

- **Counting and profiling of leukocytes.** Blood drawn from mice into a haematocrit capillary treated with heparin (Terumo, Tokyo, Japan) was collected and mixed with Turk solution and leukocytes were counted on a Neubauer haematocytometer. For profiling of the leucocyte population, blood was smeared on a glass slide, stained with May–Giemsa solution and examined under a microscope.

- **Pathology and immunohistochemistry.** Organs were fixed in 4% (v/v) formaldehyde in PBS and embedded in paraffin. Serial sections were prepared and stained with haematoxylin and eosin (H&E) solution for light microscopy or subjected to immunohistochemical staining with an antiserum to the nucleoprotein (NP) of influenza A/PR/8/34 virus. The specificity of the anti-NP antibody has been confirmed elsewhere (Iwasaki et al., 1999) and the reactivity of the antibody to H5N1 influenza viruses was confirmed by Western blotting analysis (data not shown). The staining was performed by the avidin–streptavidin–peroxidase method using diaminobenzidine as the substrate.

Results

**Virulence in mice**

The HK156 and HK483 viruses used in this study were isolated from deceased patients who were the index (3-year-old boy) and the third (13-year-old girl) cases, respectively, of the outbreak in Hong Kong in 1997 (Yuen et al., 1998) and they belong to the two different antigenic/phylogenetic groups identified among the human isolates (Suarez et al., 1998; Bender et al., 1999). In order to determine their virulence, ddY mice were infected with each virus by the nasal route. The LD<sub>50</sub> of the virus seeds to the mice were determined by inoculation of serial 10-fold dilutions of virus seeds, 10 µl per mouse (5 µl per nostril), to a set of five mice each, followed by observation for 2 weeks, and the LD<sub>50</sub> titres were compared with their plaque-forming titres. Thus, it was revealed that 200 and 5 p.f.u. of HK156 and HK483, respectively, were required to give one LD<sub>50</sub>.

The mice killed by HK156 in this assay died between day 4 and 13 post-inoculation (p.i.) and those killed by HK483 died between day 5 and 8 p.i., but there was no significant difference
between the viruses in the mean time to death at each virus load (data not shown). Severe emaciation and prostration were observed in most mice that subsequently died.

Lymphopenia and/or leukopenia were prominent without exception for the severe human cases in the clinical records (Yuen et al., 1998). Therefore, blood was drawn from three mice by venipuncture at the orbital plexus before and 4 days after infection with HK483 to compare the leukocyte count and profile in each mouse at the two dates. The leukocyte count decreased drastically in all the three mice (40, 60 and 70% decrease) and the decrease was caused mainly by depletion of lymphocytes. A similar drastic decrease in the ratio of the lymphocyte population was recognized in the blood of mice infected with HK156 at day 4 p.i. (data not shown).

**Pathological and virological findings**

In order to determine the basis of the high virulence of the viruses, mice were infected with HK156 or HK483 and their organs were subjected to pathological and immunohistochemical analyses and virus titration on days 1, 4 and 7 using sets of three mice each. Mice infected with HK156 and HK483 showed essentially similar pathological features for all of the organs we examined, except that a quantitative difference was recognized in the brain lesion. Macroscopically, on day 4, lungs of each group showed lobular consolidation; however, no significant change was observed in the heart, liver or kidney (data not shown). Histologically, the pulmonary lesions caused by both viruses were acute bronchopneumonia: desquamation of the bronchial epithelial cells, peribronchial infiltrates and enlargement of the alveolar cells were observed (Fig. 1a). These changes were first noticed on day 1 and became prominent on day 4. The thymus showed involution with depletion of lymphocytes. However, no degeneration/necrosis was observed in the tissue. No degeneration/necrosis was detected in the heart (Fig. 1g), liver, kidney, spleen, pancreas, gastrointestinal tract or lymph nodes (data not shown). Damage to endothelial cell linings was not recognized in our mouse model, although it was reported as the basis for the pathogenicity in chickens infected with HK156 (Suarez et al., 1998). The brain showed viral encephalitis on day 4, but the lesions of HK483-infected mice were far more severe than those of HK156-infected mice. Many foci of neuronal degeneration with granulocytic infiltration and with neuronophagia and slight perivascular cuffing were recognized in the brainstem and cerebrum of HK483-infected mice (Fig. 1d). Even in such mice, however, no marked inflammatory infiltrates were observed in the subarachnoid space.

In order to reveal the tissue tropism of these viruses at the cellular level, organs were subjected to immunohistochemical analysis using an antiseraum against influenza A virus NP. Again, HK156 and HK483 showed essentially similar results: on day 1, antigen-positive cells were distributed in the upper and lower respiratory tracts, where only epithelial cells such as alveolar cells and columnar epithelium of the nasal cavity and bronchi were infected and, on day 4, the infected cells increased in number in the nasal mucosa, frequently being associated with desquamation of the epithelial cells layer (Fig. 1b, c); the antigen was also detected in muscle fibres in the heart, although it was not found so frequently, and in hepatocytes in the liver, where detection was very sporadic (Fig. 1f, h). It should be noted, however, that neither inflammatory nor necrotic change was recognized in the myocardium of the heart (Fig. 1g) and that the viral antigen in the hepatocyte was located only in the nucleus (Fig. 1f). The antigen was not detected in the thymus, spleen, skin, pancreas, kidney or gastrointestinal tract (data not shown). However, many foci of antigen-positive cells were observed in adipose tissues attached to the heart, thymus, kidney, spleen and spine, being associated with necrotic changes in the adipocytes (Fig. 2a, b). Features at day 4 were fundamentally unchanged, and also in mice which survived until day 7, except a slight degenerative change found very sporadically in the heart. Viral antigen was also found in neurons and ependymal cells in sections of the brain and spinal cord (Fig. 1e).

The outstanding differences found by immunohistological comparison between the two viruses were as follows: in sections of the central nervous systems at day 4, mice infected by HK483 were rich in foci in the brainstem and cerebrum, whereas those infected by HK156 were frequently negative and, if any typical foci were found after HK156 infection, they were found only in the midbrain of mice that survived until day 7, and foci were not found in the cerebrum at all (Fig. 3a–c); HK483 produced large foci in the odontoblast layer of the dental pulp (Fig. 1i) but HK156 did not.

In order to determine quantitatively the distribution of the viruses in infected mice, organs of infected mice were subjected to virus titration by plaque assay. We chose day 4 for the titration, since the infected mice began to die thereafter. Both viruses showed the highest titre in the lung, moderate titres in the brain, heart and thymus, and only low or undetectable levels in the liver and blood. HK483 showed moderate titres in the spleen and kidney, while HK156 showed only low titres in those organs.

The titres of brains of HK483-infected mice were more than 100-fold greater than those of HK156-infected mice, consistent with the findings of the immunohistochemical analysis (Table 1). The difference between HK483 and HK156 in the degree of their invasion of the brain was shown more strikingly by an additional infection experiment, in which $10^6$ p.f.u. of virus was inoculated in a smaller volume (1 µl per nostril), with the intention of not allowing the virus to run directly into the lung in order to confine its initial replication site to the nasal cavity. Consequently, the virus was detected on day 4 in the brain of all three HK483-infected mice at high titres ($10^{5.4\pm0.7}$ p.f.u./ml), whereas the virus was detected in the brain of only one of the HK156-infected mice and the titre was very low ($10^{0.8}$ p.f.u./ml).
Fig. 1. Histological and immunohistochemical analyses of lung (a–c), brain (d, e), liver (f), heart (g, h) and dental pulp (i) obtained from mice infected with HK483 virus at day 4 p.i. Images show: degeneration and necrosis of bronchial epithelium with inflammatory infiltrate and haemorrhage around the bronchus in the interstitium (a); NP antigen in residual epithelium of bronchi (arrows) and alveolar cells (arrowheads) (b); alveolar cells with positive signals of NP antigen only in the nuclei, showing the earlier phase of the infection (arrow), or in the cytoplasm, showing the later phase (arrowhead) (c); encephalitis with degenerated neurons, infiltration of neutrophils and neuronophagia (arrows) (d); detection of NP antigen in many neurons (arrows) and some glia (arrowheads) (e); a hepatocyte with NP antigen in the nucleus (f); heart tissue showing no histological or cytological changes (g); a cluster of myocardial cells with NP antigen signal that is especially evident for the cells located in its centre (h); and odontoblasts of the dental pulp strongly positive for the antigen (i). Images were obtained by H&E staining (a, d, g) or immunostaining with anti-NP antibody (b, c, e, f, h, i). Magnification, ×90 (a, b, g), ×180 (d, e), ×360 (c, f, h) or ×135 (i).
Human H5N1 influenza virus infection in mice

Fig. 2. Adipose tissue attached to the kidney of an HK483 virus-infected mouse: a well-defined necrotic lesion stained with H&E (a) is shown and the same section shows adipocytes stained strongly by immunostaining with an anti-NP antibody (b). Magnification, × 160.

Fig. 3. Comparison of foci of viral antigen-positive cells in the brainstems of mice infected with HK156 and HK483: HK483, day 4 (a); HK156, day 4 (b); and HK156, day 7 (c). The pons is shown at a magnification of × 70.

The results of the titration of both viruses in the heart, thymus, spleen and kidney were not consistent with the immunohistology. In addition, the titres for HK156 in spleen, kidney and thymus and HK483 in thymus fluctuated from mouse to mouse (Table 1). We thought that this might be attributable, at least in part, to adipose cells associated with the organs that harboured the viruses (Fig. 2). We therefore attempted virus titration of the adipose tissues as well. Mice were infected with HK483, HK156 and PR/8 (H1N1) and the perirenal, perisplenic and mesenteric fat masses (abdominal fat), the pelvic fat body and the subcutaneous brown-fat tissue that lies on the back just behind the neck (interscapular brown fat) were subjected to titration on day 4. The virus titres were high in all the adipose tissue samples from HK483- and HK156-infected mice and, in particular, the titre for the interscapular brown fat was as high as that of the lung, while A/PR/8/34
Table 1. Virus titres, viral antigen-positive cells and degeneration/necrosis of tissues of mice infected with HK156 and HK483

Virus titres are expressed as the mean titre ± SD of 10 % (w/v) organ homogenates from three mice except for those of the brain and lung, which were from six mice of two independent experiments. Titres of less than 10 p.f.u./ml are below the limit of detection of the assay and were assigned the value 0. The frequencies of immunohistologically stained cells (antigen-positive cells) or degenerated/necrotic cells (degeneration/necrosis) found in tissue sections are scored as: +, many; +, some; ±, sporadic; −, not found. Antigen-positive cells were detected by immunohistological staining with an antiserum against influenza A virus NP.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>HK156 (log10 p.f.u./ml)</th>
<th>HK483 (log10 p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antigen-positive cells</td>
<td>Degeneration/ necrosis</td>
</tr>
<tr>
<td>Brain</td>
<td>1·8 ± 0·7</td>
<td>+</td>
</tr>
<tr>
<td>Lung</td>
<td>5·9 ± 0·5</td>
<td>+ +</td>
</tr>
<tr>
<td>Thymus</td>
<td>3·5 ± 1·4</td>
<td>−</td>
</tr>
<tr>
<td>Heart</td>
<td>3·0 ± 0·3</td>
<td>±</td>
</tr>
<tr>
<td>Liver</td>
<td>0·4 ± 0·7</td>
<td>−</td>
</tr>
<tr>
<td>Spleen</td>
<td>0·9 ± 0·9</td>
<td>−</td>
</tr>
<tr>
<td>Kidney</td>
<td>0·9 ± 1·0</td>
<td>−</td>
</tr>
<tr>
<td>Blood</td>
<td>1·3 ± 1·2</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Viral antigen was found only in the nuclei of hepatocytes.
† Involvement was found.
NA, Not applicable.

Table 2. Virus titres of fat tissues of mice infected with H5N1 influenza viruses

Virus titres are expressed as the mean titre ± SD of 10 % (w/v) tissue homogenates from groups of three mice at day 4.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Virus titre (log10 p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HK483 (H5N1)</td>
</tr>
<tr>
<td>Brown fat*</td>
<td>5·7 ± 0·5</td>
</tr>
<tr>
<td>Abdominal fat†</td>
<td>3·5 ± 0·9</td>
</tr>
<tr>
<td>Pelvic fat‡</td>
<td>4·1 ± 0·3</td>
</tr>
<tr>
<td>Lung</td>
<td>6·4 ± 0·1</td>
</tr>
</tbody>
</table>

* Interscapular brown fat.
† Fat mass attached to kidney, spleen and intestine.
‡ Fat mass in the pelvic cavity.
§ No plaques were detected.
NT, Not tested.

was not detected at all (Table 2). The titre of HK483 in the fat tissue was higher than that of HK156, and this might account for the difference in the titres for each virus detected in the spleen and kidney (Table 1).

Taken together with the histopathological findings, these results indicated that viraemia caused by HK156 and HK483 did occur in mice but that their main target organs were the lung, brain and adipose tissue. The virus loads in these three organs were followed daily for 7 days after infection with HK483 to investigate the time-course of virus growth (Fig. 4). The virus titres in the lungs were already high at day 1 and the high titre was maintained until the mice began to die. It might be noteworthy that detection of the virus in the brain was very
Discussion

We have demonstrated the basis of the virulence of two human isolates of H5N1 influenza virus in mice by a combination of virus titration and histopathology. Without the latter, one might be misled that the infection in mice was pantropic, involving many organs, as was recognized in chickens (Suarez et al., 1998). However, our study showed that the affected organs were mainly confined to the lung, brain and adipose tissue, in spite of the fact that viraemia actually occurred. This was also the case when we used BALB/c mice in the same kind of infection experiment (data not shown).

The major difference between HK483 and HK156 was in their ability to invade to central nervous system, and this was more prominent in the result of an infection experiment using a smaller inoculum volume (2 µl per mouse), intended to make the infection resemble a natural infection more closely than a larger volume (20 µl per mouse). Either or both lung and brain lesions might be responsible for the lethal outcome in mice. The severity of brain lesions may, however, account for the higher lethality of HK483, since the severity of lesions of the lungs was almost equivalent for the two viruses or even greater for HK156 and no definitive difference was found in the pathological findings on other organs (Table 1).

Gao et al. (1999) separated the H5N1 viruses isolated in Hong Kong into two groups based on the degree of virulence in mice and categorized HK156 and HK483 into the same group of highly virulent strains. However, our results demonstrated great differences in pathogenicity between the two strains: the amount of HK156 virus required to give one LD$_{50}$ in mice was greater than HK483 virus in our study. Furthermore, it was greater than that reported previously by Gubareva et al. (1998) and Gao et al. (1999) but close to that in the report by Lu et al. (1999).

The degree of lethality of HK156 might be affected by a single factor or combination of factors in each experiment system such as strains, ages of mice, volumes of inocula or passage histories of virus seeds. We have preliminary observations that, when 7-week-old BALB/c mice were infected with HK156 in 20 and 2 µl inoculum volumes, the amounts of HK156 required to give one LD$_{50}$ were about 3200 and more than 10$^{18}$ p.f.u. (end-point not determined), respectively, which demonstrates the importance of the inoculum volume in this kind of experiment.

The heart showed no pathological change in our study, or showed only slight necrosis at day 7, although the viral antigen was found in myocytes. This was not consistent with an earlier study, where BALB/c mice were used and necrosis of myofibres was described (Gao et al., 1999). The difference in the extent of the heart lesion might be attributable to the mouse strains used, since we obtained preliminary results that foci made by HK483 in the hearts of BALB/c mice were larger than those in ddY mice (data not shown).

Viral antigen was detected in adipose tissues, associated with necrotic change of this tissue. Fat necrosis of virus aetiology has been reported for several kinds of viruses: rabies virus in bats and rodents (Baer, 1975; Botvinkin et al., 1985), cytomegalovirus in rats and mice (Bruggeman et al., 1987; Price et al., 1990); Ross River virus, coxsackievirus B3 and vaccinia virus in mice (Murphy et al., 1973; Hashimoto et al., 1985; Yang et al., 1985) and Hantaan virus in suckling mice (Kurata et al., 1983). In the case of rabies virus, the virus was detected in the brown fat of wild species and it was even suggested that the fat might serve as a reservoir tissue for the virus (Baer, 1975). This is the first report that influenza virus targets adipose tissue in vivo and causes fat necrosis. High titres of virus were recovered in the fat tissue of BALB/c mice as well as ddY mice infected with HK483 and HK156 (data not shown). The pathophysiological significance of this adipotropism remains to be clarified. However, from a practical aspect, one should be reminded that contamination of the virus associated with the fat tissue should be borne in mind when interpreting the results of virus titration of organs of mice infected with the H5N1 viruses. Before this study, we had nearly concluded that HK156 and HK483 caused pantropic infection involving many organs in mice, because the viruses were actually detected in organ homogenates of infected mice in previous reports (Gao et al., 1999; Lu et al., 1999). However, it is possible that at least part of the virus detected in the homogenates might be attributed to tiny fragments of adipose tissue that happened to be associated with the excised organs. Careful pathological analyses of tissues are needed in addition to virus titration of organs in order to know whether the viruses are truly pantropic.

We thank W. L. Lim (Government Virus Unit, Queen Mary Hospital, Hong Kong) for the HK156 and HK483 viruses. We thank Ms Sato and Ms Sakurai for their technical assistance in the preparation of pathological analyses. This work was supported by a Grant for Research for Emerging and Reemerging Diseases from the Ministry of Health and Welfare, Japan.

References


Received 18 February 2000; Accepted 13 June 2000