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Short communication

Semi-quantitative detection of viral RNA in influenza A virus-infected mice for evaluation of antiviral compounds

A. Sauerbrei*, A. Ulbricht, P. Wutzler

Institute for Antiviral Chemotherapy, Friedrich-Schiller University of Jena, Winzerlaer Straße 10, D-07745 Jena, Germany

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Abstract

The aim of the study was to establish a murine model for sensitive screening of potential compounds with in vitro anti-influenza A virus activity. The evaluation in this in vivo model is based on semi-quantitative detection of viral RNA using one-step reverse transcriptase polymerase chain reaction (RT-PCR). After intranasal infection of fully-conscious mice with influenza A virus, the viral load of the respiratory tract tissues was investigated. Peaks were observed in the nasopharynx between Days 1 and 4, in the trachea on Day 4, and in the lungs between Days 4 and 7 post infection. The elimination of virus correlated with the appearance of specific serum antibodies. After 4 days of treatment with zanamivir, trachea and lungs revealed negative RT-PCR results, whereas viral load in the nasopharynx was significantly reduced. In conclusion, the virus spread in the described murine model is similar to upper respiratory tract infection with influenza virus in human. Viral load measurement by semi-quantitative detection of viral RNA allows rapid and sensitive screening of potential compounds with in vitro anti-influenza A virus activity.

Keywords: Influenza A virus; Antiviral compounds; Mouse model; Infection of respiratory tract; Reverse transcriptase polymerase chain reaction

1. Introduction

Prevention of influenza is most effectively accomplished by vaccination. Its significance, however, is limited by the low vaccination coverage, the limited effectiveness of existing vaccines and the emergence of new viral strains. Therefore, antiviral drugs could play an important role in treatment and prevention of influenza. Recently, the neuraminidase inhibitors zanamivir and oseltamivir have been shown to be active against both influenza A and B viruses and have been approved in a number of countries for treatment of human influenza virus infections (Dunn and Goa, 1999; McClellan and Perry, 2001).

Further efforts to develop anti-influenza viral drugs require the availability of suitable animal models which are equivalent to infections in human and which will be predictive of the expected clinical effects. For in vivo evaluation of potential influenza inhibitors, mouse and ferret models have been extensively used (Sidwell, 1999; Sidwell and Smee, 2000). Testing new antiviral drugs in these animal infections has a high predictive value for their clinical effectiveness. In the mouse pneumonia model, which is considered a great challenge to antiviral compounds, the efficacy of antiviral agents has been determined by lethality, weight loss and hypothermia of animals (Loosli, 1949; Sidwell, 1999; Bantia et al., 2001). In general, anti-influenza viral inhibitors should be evaluated in murine models which are similar to human influenza infections. An opportunity is provided by the mouse tracheitis model, representing an influenza virus infection of the upper respiratory tract (Ramphal et al., 1979b; Renegar, 1992; Hastings et al., 1996). In this model, complete desquamation of the tracheal epithelium has been revealed by electron microscopy within 3 days of infection (Ramphal et al., 1979b). The outcome has been assessed by isolation of infectious virus from nasal washes and/or homogenates of the lungs in cell culture followed by hemagglutination of cell supernatants. However, virus isolation in cell culture is time-consuming, needs considerable experience and has a low level of sensitivity (Liolios et al., 2001).

Thus, the objective of the present study was to infect mice intranasally with influenza A virus and induce an infection of the upper respiratory tract. The infection process was monitored by detection of viral RNA in the tissues of the nasopharynx, trachea and lungs. Thus we should be able to

* Corresponding author. Tel.: +49-36-41-657300; fax: +49-36-41-657301.
E-mail address: andreas.sauerbrei@med.uni-jena.de (A. Sauerbrei).

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establish an animal model for in vivo evaluation of potential antiviral compounds for use after routine screening of in vitro anti-influenza activity.

2. Materials and methods

2.1. Animals

Specific pathogen-free female Balb/c mice, 7 weeks old (15–17 g), obtained from Charles River Deutschland GmbH (Sulzfeld, Germany) were used in all experiments. The animals were housed six per cage. They were kept under conditions which prevented cage-to-cage infections and received food and water ad libitum.

2.2. Adaptation and preparation of virus

The influenza A virus strain Hong Kong (H3N2) was adapted by 30 in vivo passages in mice. To this end, the animals anesthetized by intraperitoneal application of 300 μl (0.6 mg) Etomidat-®Lipuro (B. Braun Melsungen AG, Melsungen, Germany) were infected intranasally with 50 μl seed virus suspension. Three to seven days post inoculation (p.i.), the mice were killed and the lungs were removed, and then homogenized to a 10% suspension in minimum essential medium (MEM). Subsequently, homogenates were frozen and thawed. The supernatant prepared by centrifugation and sterile filtration was used for additional passages in mice. After approximately five passages, the virus was cultivated in embryonated chicken eggs and titrated by the hemagglutination test. Ten-day-old eggs were infected with 300 μl supernatant diluted 1:10 to 1:100 in MEM. Following incubation for 72 h at 36 °C and 12 h at 4 °C, allantoic fluid was collected and clarified by centrifugation, before mice were infected again with 20–50 μl.

To generate a virus pool, the virus was replicated in the allantoic fluid of fertile chicken eggs after inoculation of 300 μl of stock virus diluted 1:100 in MEM. The collected allantoic fluid was stored at −80 °C. Virus stock titrated by means of plaque assay using Madin Darby canine kidney (MDCK) cells yielded 10^4 plaque forming units (PFU)/ml.

This mouse-adapted virus induced a fatal pneumonia within 4 to 7 days, if anesthetized mice were challenged by intranasal instillation of 20 μl virus suspension corresponding with 2×10^2 PFU.

2.3. Murine influenza A viral infection

Awake mice ($n = 39$) were infected intranasally without anesthetic with 10 μl virus suspensions containing 10^2 PFU influenza A virus. The animals in the control group ($n = 5$) received phosphate-buffered saline (PBS). During this procedure, the upper part of nose was held down to minimize the possibility that the virus would be swallowed or could enter the trachea directly. Clinical signs of infection such as changes of body weight, rectal temperature, behavior and fur of animals were checked daily until animals were killed. At given intervals, three mice were culled. They were anesthetized with Etomidat-®Lipuro and bled from the heart using a syringe. The animals were cut open ventrally along the median line from the xiphoid process to the point of the chin and the trachea as well as the lungs were removed aseptically. Subsequently, the head of the mouse was removed and the lower jaw was cut off. After cleavage of the head, tissue was collected from the nasopharynx using a syringe needle. The lung index expressed as the ratio of lung weight to body weight was determined (Schulman, 1968). All specimens were separately stored at −80 °C until RNA was isolated. Blood samples were centrifuged and the plasma obtained was frozen at −20 °C.

2.4. Isolation of RNA and reverse transcriptase polymerase chain reaction

The tissues of the infected mice weighed as follows: nasopharynx 16–24 mg, trachea 40–50 mg and lungs 100–140 mg. For isolation and purification of total RNA, RNeasy® Mini Kit (Qiagen, Hilden, Germany) was used. Tissues were disrupted and simultaneously homogenized in 350 μl (nasopharynx, trachea) or 600 μl lysis buffer (lungs) by use of the rotor-stator homogenizer Ultra-Turax T8 including the dispersing tool S 8 N-5 G (IKA®-Werke GmbH & Co. KG, Staufen, Germany). The efficiency of RNA isolation from nasopharynx, trachea and lungs was examined spectrophotometrically and 0.3 μg RNA were used for further investigations. One-step reverse transcriptase polymerase chain reaction (RT-PCR) (Roche Diagnostics, Mannheim, Germany) was performed with oligonucleotide primers selected from the highly conserved nucleotide sequences of influenza A viral RNA segment 8 (Claas et al., 1992) coding for non-structural proteins (primer 1, nucleotides 467-486: 5′-AAGGGCTTTCACCGAAGAGG-3′, primer 2, nucleotides 637-656: 5′-CCCATTCTCATTACTGCTTC-3′, amplified product 190 bp). To assess the specificity, RNA from influenza B virus served as controls. Inhibition of RT-PCR by zanamivir as well as varying efficiency using RNA targets from different organs were excluded by internal controls. After staining with ethidium bromide, amplified DNA fragments were separated by gel electrophoresis in 2% agarose and semi-quantitatively analyzed under UV light by comparing signal intensity with those of positive and negative controls. Visible bands of DNA were assigned the following score by which the viral load of the tissues harvested was expressed: (i) very strongly positive (4), (ii) strongly positive (3), (iii) positive (2), (iv) weakly positive (1), (v) negative (0) (Fig. 2). For each group of three mice, the mean score of viral load was calculated. Finally, PCR products were randomly specified by Southern blot hybridization on nylon membranes using an internal oligonucleotide of the

described primer pair as probe (nucleotides 564-593: 5′-GTCCTCATCGGAGGACTTGAATGGAATGAT-3′). The sensitivity of RT-PCR was assessed with 10^{-3} PFU influenza A virus per ml (Fig. 2).

To evaluate statistical differences between groups of animals, the data were compared using Student's *t*-test (*P*-values subject to a significance level of 5%).

2.5. Detection of virus-specific antibodies

Mouse sera were tested for the presence of antibodies to non-selected structural proteins of the whole influenza A virus by indirect fluorescence antibody technique. Sera were initially diluted 1 in 5 and incubated with fixed influenza A virus strain Hong Kong (H3N2)-infected MDCK cells. Poly-specific fluorescein-labeled rabbit antimouse immunoglobulins (Dako, Glostrup, Denmark) served as conjugate. Sera were considered positive if a specific green fluorescence of virus-infected cells could be observed under the fluorescence microscope.

2.6. Treatment of mice

For treatment, mice infected as described before were treated intranasally with 20 μl of 0.5% zanamivir (4-guanidino-Neu5Ac2en, GG 167, GlaxoSmithKline, UK) corresponding to approximately 6 mg/kg body weight/dose or placebo (PBS) three times daily over 4 days. The first doses were administered 60 min before, 10 or 60 min after virus exposure. Each group consisted of eight mice. The tissue samples were collected at 12 h after the last administration of substance.

3. Results

3.1. Clinical outcome of infection

All mice intranasally infected with influenza A virus remained symptom-free and survived. By Day 4, after viral exposure, when clinical signs of murine influenza pneumonia are usually observed (Sidwell, 1999), the mice had not lost weight (mean body weights: day of inoculation 16.4 g, 4 days p.i. 16.3 g) and there were no differences relating to the rectal temperatures (mean rectal temperatures: day of inoculation 38.0 °C, 4 days p.i. 37.9 °C). Changes in behavior, especially tendencies to huddle and diminished vitality, or ruffled fur, were not observed. The mean lung index of the virus-infected mice (0.8) was not different from that of the control animals (0.8).

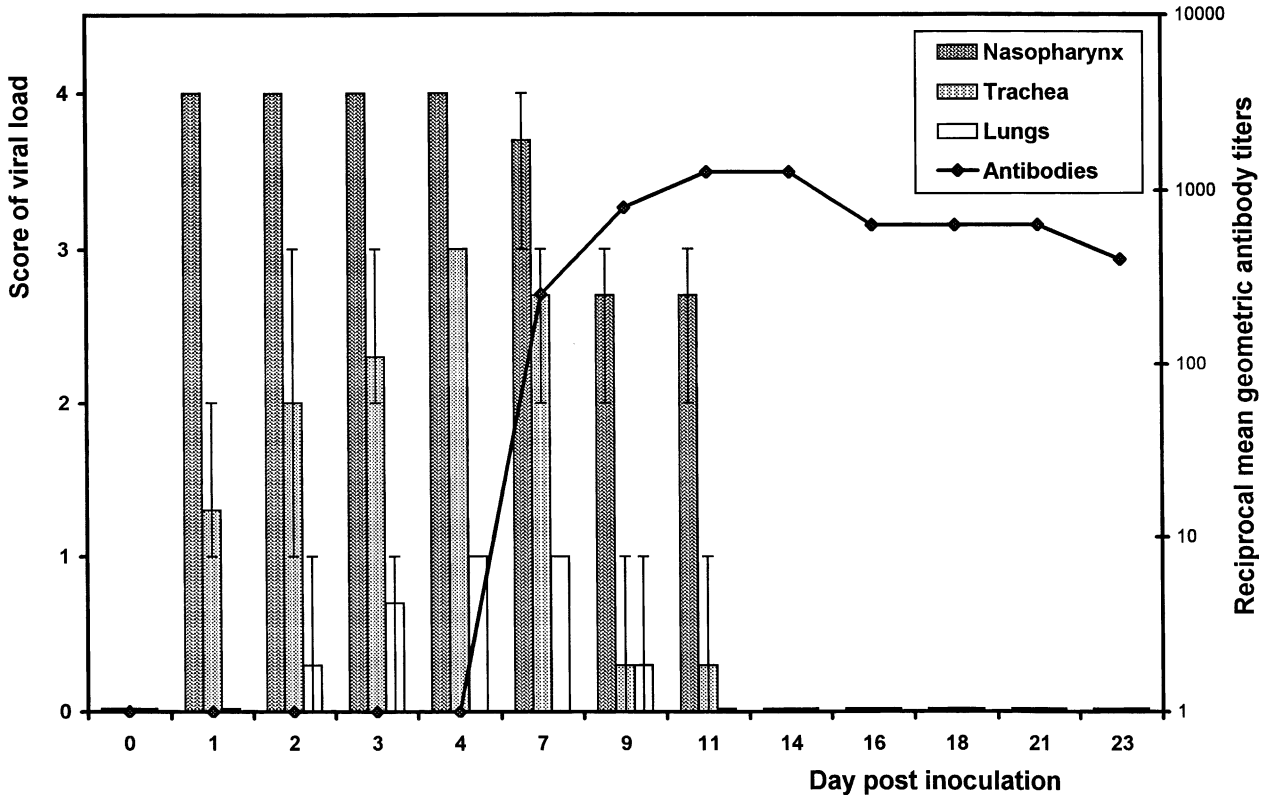

Fig. 1. Semi-quantitative analysis of the viral load by reverse transcriptase polymerase chain reaction (RT-PCR) in the tissues of nasopharynx, trachea and lungs and appearance of virus-specific antibodies in mice after intranasal infection with influenza A virus ($n = 3$). Visible bands of reverse transcribed DNA were assigned the following score of viral load: (i) very strongly positive (4), (ii) strongly positive (3), (iii) positive (2), (iv) weakly positive (1), (v) negative (0).

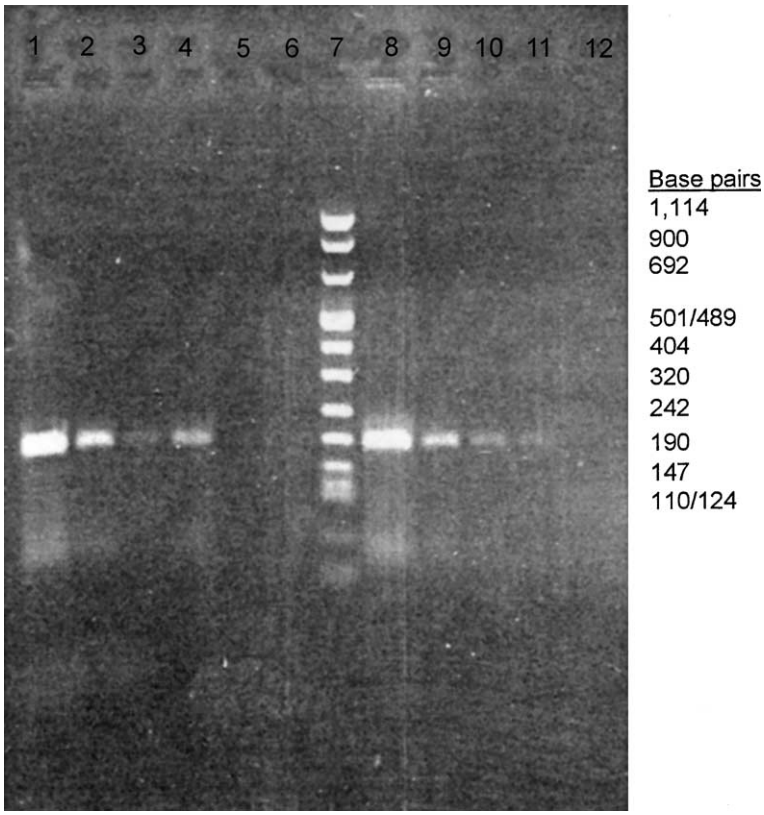

Fig. 2. Reverse transcriptase polymerase chain reaction (RT-PCR) and gel electrophoresis for detection of influenza A viral RNA. Lanes 1–3: infected mouse on Day 4 post inoculation (p.i.), 1: nasopharynx, 2: trachea, 3: lungs; 4–6: infected mouse after 4-day-treatment with zanamivir, first dose 10 min post infection, 4: nasopharynx, 5: trachea, 6: lungs; 7: molecular weight standard VIII Roche Diagnostics; 8–12: controls, 8: 1 plaque forming units (PFU)/ml influenza A virus (score 4—very strongly positive), 9: 10^{-1} PFU/ml (score 3—strongly positive), 10: 10^{-2} PFU/ml (score 2—positive), 11: 10^{-3} PFU/ml (score 1—weakly positive), 12: 10^{-4} PFU/ml (score 0—negative).

3.2. Monitoring the viral load and antibody detection

At 2 h p.i., no influenza A viral RNA could be detected by RT-PCR in the nasopharynx, trachea and lungs of the inoculated mice (Fig. 1). Viral RNA could be amplified (score of virus load >1) in specimens of nasopharynx between Days 1 and 11, and in the trachea between Days 1 and 7 after infection, respectively. Weakly positive results (score of virus load = 1) could be obtained in the lungs from those animals which were killed between the 4th and 7th day (Fig. 1). In the nasopharynx, the highest level of viral load was detected between Days 1 and 4 (score 4), in the trachea (score 3) on Day 4, and in the lungs (score 1) between Days 4 and 7 post infection. All tissues harvested on Day 14 or later, revealed negative PCR results. Virus-specific antibodies could be measured from Day 7 after infection. The highest geometric mean antibody titer of 1:1280 was reached on Day 11 p.i. (Fig. 1).

3.3. Treatment by zanamivir

After 4 days of treatment with zanamivir, viral RNA could only be detected in the nasopharynx while the trachea as well as the lungs were negative (Figs. 2 and 3). The viral load obtained from nasopharyngeal specimens was significantly diminished ($P = 0.05$) if compared with control animals without antiviral treatment (score = 4). When the first dose of zanamivir was given 60 min before inoculation, a reduction of score from 4 to 3.5 was achieved while the score was 3.3 and 3.0, if zanamivir was first administered at 10 or 60 min p.i., respectively.

4. Discussion

In this study, a murine influenza A virus model that mimics the pathogenesis of local viral infections of the human upper respiratory tract was established. The spread of virus seems to be comparable with that of the tracheitis model described by Ramphal et al. (1979b). However, neither clinical nor macroscopic signs of manifested tracheitis were evident in our experiments. Histology and electron microscopy were not performed. Because the mice did not show symptoms typical for influenza, e.g. pneumonia, the infection process was monitored by measuring the viral load in the tissues of the nasopharynx, trachea and lungs. In other murine exper-

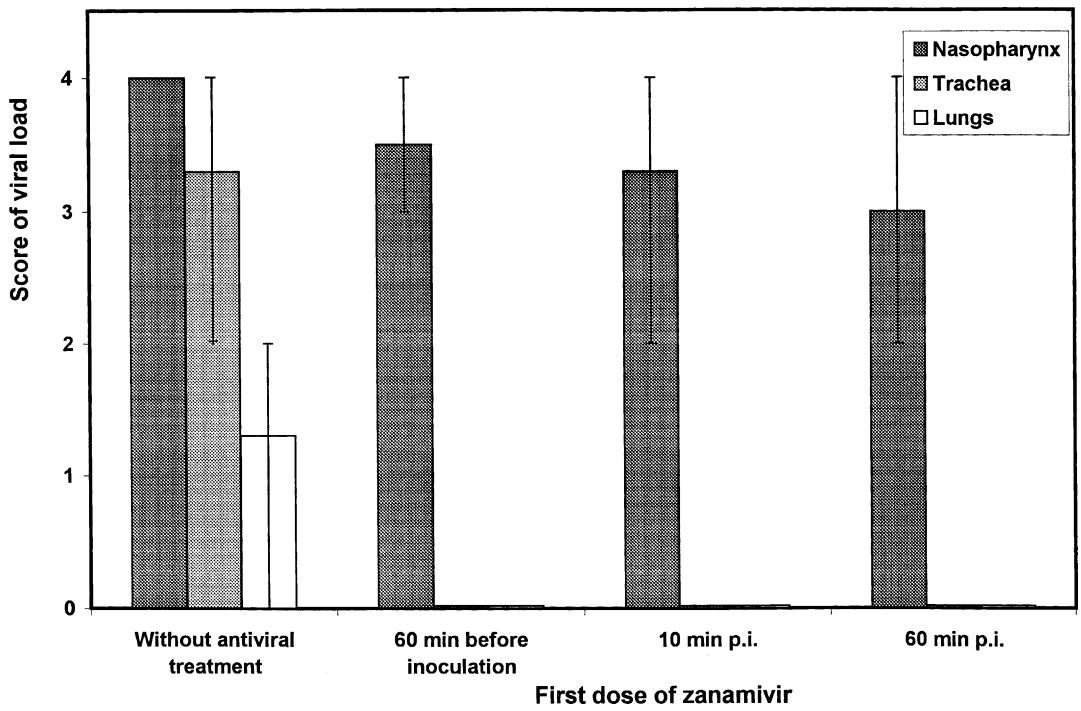

Fig. 3. Semi-quantitative analysis of the viral load by reverse transcriptase polymerase chain reaction (RT-PCR) in the tissues of nasopharynx, trachea and lungs of mice on Day 4 after intranasal infection with influenza A virus and the 4-day treatment with zanamivir ($n = 8$), p.i.—post inoculation.

iments, influenza virus has been isolated and subsequently titrated in embryonated chicken eggs (Yetter et al., 1980; Novak et al., 1993) or MDCK cells (Hastings et al., 1996) from pooled nasal washes and/or animal tissues. Since these methods are time-consuming, need considerable experience and have a reduced sensitivity (Liolios et al., 2001), they are less convenient for routine screening of potential antiviral compounds in mice. Thus, the outcome of infection was assessed by highly sensitive semi-quantitative amplification of reverse-transcribed viral DNA. To our knowledge, PCR amplification has only been used in the studies of Mori et al. (1995) to investigate the pathogenesis of influenza A virus infection in mice.

A limitation of the RT-PCR we used is that, this method is valid only for the comparison of nucleic acid levels between samples but not for the determination of absolute amounts of viral RNA (Vu et al., 2000). Virus isolation techniques seem to allow a better quantitative evaluation of results. In addition, the PCR does not give information about the infectious viral load which may also have significance for the comparison of drug potencies. As reference, a non-standardized sample with a defined viral concentration expressed in PFU was used, whereas a reference with an absolute amount of influenza viral RNA was not available. This means that the RT-PCR sensitivity assessed in our study with 10^{-3} PFU influenza A virus per ml depends on the ratio of the number of infectious particles to the total number of physical particles in the viral preparation used. Finally, more objective quantification of PCR products can be achieved by densitometric scanning of Southern blots or dot-blots.

In agreement with previous studies, the viral load in the nasopharynx appeared to peak between 24 and 96 h post infection (Novak et al., 1993; Hastings et al., 1996). Highest detectable levels of viral RNA in the trachea and lungs were not found until 96 h post exposure. This underlines the fact that virus replication in the nasopharyngeal tract precedes the viral spread to the trachea. In the lungs, only small amounts of viral RNA could be detected and there were no clinical or macroscopic signs indicative of pneumonia. The elimination of virus in the nasopharynx up to the 14th day correlated with the appearance of virus-specific serum antibodies. Presumably, serum antibodies reflecting T cell immunity limited viral replication in the lung but seemed to have no significant effect on tracheal and nasopharyngeal virus titers (Ramphal et al., 1979a). To protect the upper respiratory tract from intranasal viral challenge, secretory immunity (specifically IgA) plays a more significant role (Renegar et al., 2001). The findings suggest that influenza virus infection began in nasal tissue and progressed to the upper respiratory tract. This pathogenesis is comparable with that of typical human influenza infections.

In similar experiments, high virus titers were detected in the lungs at 48 h post infection (Hastings et al., 1996) presumably caused by higher inoculum doses of virus (Novak et al., 1993). This may include a direct infection of the lung and so a higher risk of pneumonia combined with weight loss, followed by death of the animals. In contrast to the infection model described in the present paper, the intranasal instillation of influenza virus into the nares of anesthetized mice or exposure to a small-particle aerosol leads to clini-

cal signs in animals such as weight loss, hypothermia and behavioral changes, pneumonia and death (Renegar, 1992; Sidwell, 1999). However, the pathogenesis of infection and viral spread are not related to typical influenza virus infection in humans. Moreover, it must be considered that the mouse does not develop the characteristic human symptoms of influenza such as fever and nasal discharge (Sidwell, 1999). Both the pneumonia and respiratory tract infection models require virus strains which have to be highly adapted to mice. In particular, recent clinical isolates of influenza A virus without prior adaptation are known to induce a toxic pneumonitis in the absence of viral replication (Barker and Hoyle, 1972) and non-adapted H5N1 isolates may cause severe generalized infections in BALB/c mice (Gubareva et al., 1998).

The present murine model infection of the respiratory tract accompanied by monitoring the viral load in the respiratory tract using PCR can be recommended for rapid and sensitive screening of compounds with potential anti-influenza A virus activity. So, in mice treated intranasally with zanamivir, viral RNA was uniformly not detected in the trachea as well as the lungs and the viral load in the nasopharynx was decreased when first doses were administered 60 min before, or 10 or 60 min after challenge of virus. These results are in agreement with other studies that have shown a high inhibitory prophylactic and therapeutic activity of zanamivir against murine influenza pneumonia (Ryan et al., 1994; Fenton et al., 1999). Additionally, our findings demonstrate the post-exposure efficacy of zanamivir in influenza A infections of mice. To confirm this effect, zanamivir should also be tested at later time-points than 60 min after inoculation of virus because this drug is usually given within 1 to 2 days after onset of illness. Test compounds can easily be administered to fully conscious animals by intranasal or other routes of application. For intranasal instillation, it should be taken into account that repeated administration of fluids into the lung exacerbates the infection (Takano et al., 1963). This effect may be circumvented if the therapeutic compound is administered by small particle aerosol (Wyde et al., 1986). The viral load of the respiratory tract can be estimated by highly sensitive and specific one-step RT-PCR, the results of which are available within one day. Because of the preparation and additional purification of RNA, test compounds are removed from specimens and, therefore, cannot interfere with the reverse transcription process or amplification of the viral DNA products. The comparability of viral spread in the present murine influenza A virus infection with that of typical influenza in humans, coupled with the advantages of PCR makes this model a valuable tool to screen antiviral compounds for their effectiveness in vivo.

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