Total viral genome copies and virus–Ig complexes after infection with influenza virus in the nasal secretions of immunized mice

Tomoki Yoshikawa,1,2 Keiko Matsuo,1 Kazutoshi Matsuo,3 Yujiro Suzuki,4 Akio Nomoto,2 Shin-ichi Tamura,1,5 Takeshi Kurata1 and Tetsutaro Sata1

1Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
2Department of Microbiology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
3Feed Division, Livestock Industry Department Agricultural Production Bureau Ministry of Agriculture, Forestry and Fisheries, 1-2-1 Kasumigaseki, Chiyoda-ku, Tokyo 100-8950, Japan
4Research Center for Biologicals, Kitasato Institute, 6-111 Arai, Kitamoto-shi, Saitama 364-0026, Japan
5Laboratory of Prevention of Viral Diseases, Research Foundation for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

Correspondence
Tomoki Yoshikawa
ytomoki@nih.go.jp

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INTRODUCTION

Infection with natural influenza A virus provides cross-protection against drift viruses within a subtype in mice and humans (Schulman & Kilbourne, 1965; Couch & Kasel, 1983). The ability to provide cross-protection against drift viruses correlates with the induction of cross-reacting secretory IgA antibodies (Abs) in the respiratory tract and not with serum IgG or cytotoxic T lymphocyte (CTL) responses (Liew et al., 1984; Murphy & Clements, 1989; Asahi et al., 2002).

The molecular mechanism of protection against virus infection by Abs is known as neutralization, a process in which binding of Abs to virus can result in decreased viral infectivity. IgA neutralizes viral infectivity by forming virus–Ig complexes, which prevents either the attachment to or penetration into cultured epithelial cells by the virus or neutralizes the virus within the cultured cell (Outlaw & Dimmock, 1990, 1991; Armstrong & Dimmock, 1992). Secretory IgA has a higher in vitro neutralization efficiency than IgG due to its polymeric nature (Renegar et al., 1998). An in vivo study showed that virus–IgA and virus–IgM immune complexes were detected in the faeces of newborn piglets infected with rotavirus at the time of virus clearance (Corthier & Vannier, 1983). This finding supports the concept that Abs neutralize viral infectivity in vivo by
forming virus–Ig complexes that result in clearance of infectious virus. The mechanism of virus–Ig complex formation in vivo and clearance of infectious virus from the respiratory tract is unknown.

The detection of influenza virus RNA in patients with respiratory tract infections using real-time RT-PCR has been developed recently (van Elden et al., 2001). Another recently developed method is the use of immunocapture RT-PCR, which has been used to detect hepatitis C virus (HCV) particles bound to different anti-HCV Ig isotypes: viral particles were captured using specific Abs and then amplified (Peng et al., 2001). These highly sensitive methods are useful tools for measuring the total number of influenza virus particles and were used in this study to ascertain the number of viruses in influenza virus–IgA complexes in nasal secretions of mice. The role of the virus–IgA complexes in providing cross-protection against variant viral infections was investigated.

In the present study we investigated the kinetics of total influenza virus particle production and that of virus within virus–Ig complexes after infection with A/PR8 (H1N1), and analysed the kinetics of infectious virus (p.f.u.) production as an index of infectious virus levels in the nasal secretions of naive mice and mice immunized 4 weeks previously with the A/PR8, A/Yamagata (H1N1), A/Guizhou (H3N2) and B/Ibaraki strains of influenza virus. The total virus number and the number of viruses within the immune complexes, captured using anti-mouse Ig-coated plates, were estimated on the basis of a viral genome copy number determined by quantitative RT-PCR (Q-PCR) (Peng et al., 2001; van Elden et al., 2001). The results showed that the total virus number, which was $10^{10}$ fold higher than the number of infectious virus particles, correlated with the number of p.f.u. identified in nasal secretions of naive and immunized mice. Analysis of the number of p.f.u. and the total virus number revealed earlier virus elimination from the nasal area in the immunized mice than in the naive mice. The rate of virus elimination increased with the level of antigenic relatedness between the immunizing and challenging viruses. The rate of virus elimination in immunized mice correlated with the level of A/PR8 virus-reactive Abs. Virus elimination was accompanied by the formation of virus–Ig complexes shortly after infection. These results suggested that the formation of virus–Ig complexes is involved in the rapid clearance of virus from the upper respiratory tract observed in immunized mice. Based on the results of this study, we have proposed a four-stage process of virus elimination to help explain the mechanism of virus–Ig complex formation in vivo and clearance of infectious virus from the respiratory tract.

**METHODS**

**Viruses.** These experiments used the following immunologically different (Tamura et al., 1992) mouse-adapted influenza virus strains: A/PR/8/34 (A/PR8; H1N1), A/Yamagata/120/86 (A/Yamagata; H1N1), A/Guizhou/54/89 (H3N2) x A/PR8 (A/Guizhou-X) and B/Ibaraki/2/85 (B/Ibaraki).

**Infection.** BALB/c mice (female, 6 weeks old; Japan SLC, Hamamatsu) were anaesthetized by intraperitoneal injection of amobarbital sodium (0.25 ml of a 1 µg ml⁻¹ solution) and immunized by intranasal application of 2 µl virus suspension. The dose-restricted volume (2 µl) of the virus suspension induced a transient infection localized to the upper respiratory tract and was not lethal (Yetter et al., 1980; Tamura et al., 1996). The following doses of virus were used to induce an appropriate level of individual anti-influenza virus Ab response: 7.4× 10⁴ p.f.u. for A/PR8, 4.0× 10⁶ p.f.u. for A/Yamagata, 4.0× 10³ p.f.u. for A/Guizhou-X and 1.1× 10⁵ p.f.u. for B/Ibaraki. Four weeks after immunization, mice were infected with 7.4× 10⁵ p.f.u. A/PR8 virus.

**Virus inactivation.** The A/PR8 virus was inactivated by UV irradiation and treated nine times with an autocross-link mode (120 000 µl cm⁻² once) using a Stratmlinker UV cross-linker 1800 (Stratagene). The UV treatment of live A/PR8 virus, capable of producing 7.4× 10⁵ p.f.u. per mouse in Madin–Darby canine kidney (MDCK) cell culture, resulted in the complete loss of infectivity. This treatment resulted in only a slight reduction in total viral genome copy number from 7.4× 10⁷ to 1.3× 10⁷.

**Nasal wash specimens.** Mice were anaesthetized and bled from the heart. The nasal wash was collected by washing the nasal cavity three times with the same 1 ml of PBS containing 0.1% BSA (Tamara et al., 1992).

**Ab titration.** IgA and IgG against haemagglutinin (HA) molecules purified from the A/PR8, A/Yamagata, A/Guizhou-X and B/Ibaraki viruses (Phelan et al., 1980) or those against whole virus particles of the A/PR8 virus were measured by ELISA as described previously (Tamura et al., 1996). Briefly, ELISA was conducted sequentially from the solid phase (ELISA plate; Costar) with a sequence of reagents consisting of the following: (i) purified HA molecules or rabbit anti-A/PR8; (ii) nasal wash; (iii) goat anti-mouse IgA (z-chain-specific; Southern Biotechnology Associates) or goat anti-mouse IgG (z-chain-specific; Jackson ImmunoResearch Laboratories) conjugated with biotin; (iv) streptavidin conjugated with alkaline phosphatase (Invitrogen Corporation) or streptavidin conjugated with β-galactosidase (Invitrogen Corporation); and (v) p-nitrophenylphosphate for alkaline phosphatase detection or 4-methylumbilliferyl-β-D-galactoside for β-galactosidase detection. The chromogen produced by alkaline phosphatase was measured for absorbance at 405 nm with a Labsystems Multiskan MS (Dainippon Pharmaceutical). The fluorescence produced by β-galactosidase was measured for excitation at 355 nm and emission at 460 nm with a Labsystems Fluoroskan II (Dainippon Pharmaceutical). Both A/PR8 HA-specific and A/PR8 virus-specific Ab levels were determined using a twofold serial dilution of purified HA-specific polyclonal IgA or HA-specific monoclonal IgG (starting at 160 ng ml⁻¹ each) as a standard. The Ab concentration of an unknown specimen was determined from the standard regression curve constructed for each assay. HA-specific Ab levels for viruses other than A/PR8 virus were determined using a twofold serial dilution of unknown nasal wash specimen, which was placed on the ELISA plate coated with the virus-specific HA molecules and expressed as the highest nasal wash dilution giving a positive reaction. The cut-off value was set as the mean +2 SD of a twofold serial dilution of pre-immune nasal wash specimens. The neutralization test was performed according to a standard method and the titre was represented by the serum dilution giving a 50% reduction in number of p.f.u.

**Plaque assay.** Serial 10-fold dilutions of the nasal wash in PBS with 0.1% BSA were prepared and 0.2 ml aliquots were added to
MDCK cells in a 6-well plate. After 1 h of adsorption, each well was overlaid with 2 ml 0·6 % agar medium (Tobita et al., 1975; Tobita, 1975). After 2 days incubation in a CO2 incubator, the plaques were counted. The viral titre was expressed as p.f.u. ml⁻¹ and represented by the mean ± SD of specimens collected from three mice of each group. Neutralization tests were carried out as follows. The A/PR8 virus suspension contained 50 p.f.u. in 0·1 ml 1 % BSA in PBS and was mixed with equal volumes of serial dilutions of antisera and allowed to react at 37°C for 1 h. Then 0·2 ml of the mixture was added to the MDCK culture in a 6-well plate, left to adsorb at 37°C for 30 min, and washed with 5 ml PBS before adding the overlay for the standard plaque assay.

Electron microscopy (EM) particle counts of influenza virus. Virus particles were counted by the loop-drop method (Watson et al., 1963; Shiraki et al., 1991). Briefly, the virus solution was mixed with a standard latex particle solution (Stadex, 100 nm diameter; JSR Corporation). The mixture was placed on a grid and stained with 1 % neutral phosphotungstate. The number of virus and latex particles was counted using an electron microscope and the virus particle count was determined by comparison with the latex particle count in the standard solution.

Extraction of viral RNA from virus–Ig complexes in the nasal wash. Total viral RNA in the nasal wash (250 µl) was extracted with Trizol (Invitrogen Corporation) according to the manufacturer’s instructions. The virus–Ig complexes between virus and antiviral IgA, IgG or IgA/G(M) Abs in the nasal wash were separated as reported by Peng et al. (2001). Briefly, goat anti-mouse IgA (x-chain-specific; Southern Biotechnology Associates), goat anti-mouse IgG (γ-chain-specific; Southern Biotechnology Associates) or goat anti-mouse IgA/G(M) (Zymed) was coupled to ELISA plates (Costar). After washing with 0·05 % Tween 20 in PBS (PBS-Tween) and blocking with 1 % BSA in PBS, the plates were incubated with 50 µl nasal wash to capture the immune complexes present in the wash. After washing with PBS-Tween, viral RNA was extracted by incubating with 20 µl TE buffer (pH 8·0) and 40 µl Trizol according to the manufacturer’s instructions.

Quantification of viral matrix protein gene copies. Extracted viral RNA was reverse-transcribed into cDNA using a Sensiscript RT kit (Qiagen) containing a primer to the nucleotide sequence of the matrix protein (M1) gene (van Elden et al., 2001). The cDNA was amplified with 20 µl of a PCR mixture containing 2 µl cDNA, 2 µl LightCycler DNA Master Hybridization Probes (Roche Diagnostics), 5 mM MgCl₂, 900 nM M1 gene-specific primers (INFA-1 and INFA-2) and 200 nM M1 gene-specific TaqMan probe (INFA probe) (van Elden et al., 2001). Amplification and detection of cDNA were performed using a real-time Q-PCR system (LightCycler; Roche Diagnostics) under the following conditions: 10 min at 95°C to activate the DNA polymerase, followed by 45 cycles of 0 s at 95°C, 5 s at 55°C and 10 s at 72°C. Standard RNA was synthesized from plasmid DNA encoding the A/PR8 M1 gene by T7 RNA polymerase (Ribob Max Large Scale RNA Production System – T7; Promega) and used as the standard to calculate the copy number after spectrophotometric determination of RNA concentration.

RESULTS

Correlation of virus genome copy number by Q-PCR and EM

The viral genome copy number measured by the Q-PCR method was compared with the viral particle counts determined by EM using the A/PR8 virus, which is capable of producing 7·4 × 10⁵ p.f.u. per mouse in MDCK cell culture. Assessment of viral genome copy number by Q-PCR revealed that 61 viral genome copies were required for the production of 1 p.f.u., while EM analysis showed that 23 viral particles were required. Thus, these two methods produced comparable results for the determination of viral genome copy number as a measure of total viral particles. It was concluded that 20–60 viral particles were required to produce one infectious virus.

Specificity of the detection system for immune complexes

The specificity of the detection system for immune complexes was examined. First, 10-fold serial dilutions of immune complexes (diluted from 1 : 100 to 1 : 10⁶) formed between anti-A/PR8 HA mAb (160 ng ml⁻¹) and the A/PR8 virus (7·4 × 10⁵ p.f.u. per mouse in MDCK cell culture) were incubated with goat anti-mouse IgG-coated ELISA plates. The viral genome copy number in the total RNA, extracted from the virus–Ig complexes, was then measured by Q-PCR. As a control, serial dilutions of the complexes were incubated with BSA-coated plates. As another control, the virus number was measured for the total RNA extracted directly from the diluted viruses, without prior incubation with goat anti-mouse IgG-coated plates. The number of viral genome copies recovered from the immune complexes decreased in a linear fashion with dilutions of the immune complexes, both represented on a log scale, when the immune complexes contained more than 10⁵ viral genome copies (data not shown). Thus, virus could be recovered completely from the immune complexes bound to goat anti-mouse IgG-coated plates. In addition, non-specific binding of immune complexes to BSA-coated plates, which corresponded to approximately 0·1–0·01 % of the viral genome copy number, was observed at dilutions of the immune complexes that contained more than 10⁵ viral genome copies (data not shown). This result showed that the viral genomes in the virus–Ig complexes could be detected specifically by Q-PCR.

Next, 10-fold serial dilutions of anti-A/PR8 HA mAb (10 µg ml⁻¹) (diluted up to 1 : 10⁶) were incubated with the A/PR8 virus (1·1 × 10⁵ copies ml⁻¹, 2·2 × 10⁵ p.f.u. ml⁻¹) to form immune complexes and incubated with goat anti-mouse IgG-coated ELISA plates. The viral genome copy number in the virus–Ig complexes was measured by Q-PCR and shown to increase in the presence of 10⁻² ng mAb ml⁻¹, reaching a plateau of 10⁴⁻¹⁰⁵ copies in the range of 1 ng to 10 µg mAb ml⁻¹ with a low peak at 100 ng mAb ml⁻¹ (data not shown). Thus, a high viral copy number was involved in immune complex formation for a wide range of antibody concentrations. This made it difficult to detect slight differences in virus number within the immune complexes, depending on the level of antibody. On the other hand, an infectious virus response of 10⁴ p.f.u. ml⁻¹ was produced in MDCK cell culture after incubation of the virus with 0·001–1 ng mAb ml⁻¹. This response decreased when 100 ng mAb ml⁻¹ was used and disappeared at 10 µg mAb ml⁻¹ (data not shown). In
addition, the supernatant collected from the plate after incubation of the mixture of the virus and the mAb (100 ng ml⁻¹) with the anti-mouse IgG-coated ELISA plate decreased the ability to produce infectious virus (1/10 the number of p.f.u. of the mixture). These results suggested that virus–Ig complex formation is involved in the reduction in the amount of infectious virus produced.

**Number of p.f.u., total viral genome copy number and virus–Ig complex formation after infection with A/PR8 virus in naive mice and mice previously immunized with A/PR8 virus**

Following A/PR8 virus infection of naive mice and mice immunized 4 weeks previously with the A/PR8 virus, the kinetics of infectious virus and total viral genome copy numbers were examined in nasal secretions. In the naive mice, the number of p.f.u. after infection decreased rapidly from 0 to 3 h, gradually increased from 3 to 12 h, peaked on day 3 at 10⁴ p.f.u. (ml nasal wash)⁻¹ and declined thereafter (Fig. 1a and b). Similarly, the total virus number decreased rapidly within 3 h of infection, then increased to a peak on day 3 with 10⁸ copies (ml nasal wash)⁻¹ and declined thereafter (Fig. 1c and d). In the immunized mice, the number of infectious virus particles decreased rapidly from 0 to 3 h, increased to a peak at 12 h at 10² p.f.u. (ml nasal wash)⁻¹ and then disappeared within 24 h (Fig. 1a and b). The total virus number decreased rapidly from 0 to 3 h, increased to a peak of 10⁶ copies at 12 h, then disappeared on day 5 (Fig. 1c and d). From 3 to 12 h after infection, the number of infectious virus particles in the immunized mice [10 p.f.u. (ml nasal wash)⁻¹] was lower than observed in the naive mice [10⁵ p.f.u. (ml nasal wash)⁻¹], although the total virus number in immunized and naive mice was almost the same. In summary, the total virus number was 10³–10⁴-fold higher than the number of infectious particles in both the naive and immunized mice, and total virus persisted in nasal secretions 4–6 days longer than the infectious virus. In the immunized mice, the challenge virus was eliminated within 24 h based on the number of infectious particles and within 5 days based on the total virus number. In naive mice, challenge virus elimination occurred 8 days later based on the number of infectious particles and 10 days later based on the total virus number (Fig. 1a and d, and data not shown).

To examine the kinetics of total virus number after administration of non-infectious virus as a control, naive mice and mice immunized 4 weeks previously with A/PR8 virus were exposed to UV-inactivated A/PR8 virus equivalent to the amount of infectious A/PR8 producing a viral titre of 7·4 × 10⁵ p.f.u. (ml nasal wash)⁻¹ (Fig. 1c). In both the naive and immunized mice, inactivated virus was eliminated rapidly, with the lowest level of total virus number, approximately 10²–10³ copies, recorded 12 h after infection. There was a large difference between the total virus number after administration with infectious virus (10⁶ copies) and inactivated virus (10⁴ copies). This discrepancy could be explained by the hypothesis that the increase in virus number following release of progeny virus from infected epithelial cells in naive and immunized mice is involved in the change in total virus number observed from 3 to 12 h.

The early elimination of challenge virus observed in immunized mice compared with naive mice (Fig. 1a and b) could be explained by the presence of high levels of nasal anti-A/PR8 virus or HA-specific Abs and neutralization Abs (Table 1). To confirm this, the viral genome copy number within virus–Ig complexes after infection with A/PR8 virus was examined in the nasal secretions of immunized mice (Fig. 1e). The number of viral genome copies bound to the specific IgA, IgG or Ig(A, G and M) Abs was detected immediately after infection. Viral genome copy number decreased within 3 h, increased to a peak at 12 h and then declined slowly up to 24 h. The virus number within virus–IgA complexes (Fig. 1e) was similar to that within virus–IgG or virus–Ig(A/G/M) complexes (data not shown). We also observed that the change in the number of viruses within virus–IgA complexes was slightly lower than the total virus number. These results suggested that homologous challenge virus was captured by specific Abs immediately after infection in A/PR8 virus-immunized mice, resulting in the formation of virus–Ig complexes (Fig. 1a and d).

**Infectious virus, total viral genome copies and viral genome copies within virus–Ig complexes after challenge infection with A/PR8 virus in mice previously immunized with different viruses**

The kinetics of the infectious virus, total virus number and the number of viruses within virus–Ig complexes after challenge infection with A/PR8 virus (H1N1) was also examined in the nasal secretions of mice immunized 4 weeks previously with a different H1N1 subtype virus (A/Yamagata; 4·0 × 10⁶ p.f.u.), an H3N2 subtype virus (A/Guizhou-X; 4·0 × 10⁷ p.f.u.) and a different type virus (B/Ibaraki; 1·0 × 10⁴ p.f.u.) (Fig. 1). Based on the amount of infectious virus detected, challenge virus was eliminated from the nasal secretions 6 and 4 days earlier, respectively, in A/Yamagata and the A/Guizhou-X virus-immunized mice than in naive mice. This rate of elimination was 2 and 4 days slower, respectively, than was observed in the A/PR8 virus-immunized mice (Fig. 1a and b). The total virus number paralleled the number of infectious virus particles in both A/Yamagata and A/Guizhou-X virus-immunized mice with a peak at 12 h followed by a decline (Fig. 1c and d). Likewise, the kinetics of infectious virus production and total virus number in the B/Ibaraki virus-immunized and naive mice was equivalent (Fig. 1a–d). Thus, the challenge virus in the A/Yamagata and A/Guizhou-X virus-immunized mice was eliminated from the nasal secretions earlier than in naive mice, based on either the production of infectious virus or the total genome copy number, but at a slower rate than in A/PR8 virus-immunized mice. Consistent with these results,
A/PR8 virus cross-reactive anti-virus and anti-HA IgA or IgG Abs (Table 1), as well as A/Yamagata- and A/Guizhou-X-specific anti-HA IgA and IgG Abs (Table 2), were produced in A/Yamagata and A/Guizhou-X virus-immunized mice. The level of cross-reactive Abs was lower in the A/Guizhou-X virus-immunized mice than in the A/Yamagata virus-immunized mice.

The presence of the A/PR8 virus cross-reactive Abs in the A/Yamagata and the A/Guizhou-X virus-immunized mice was accompanied by the development of virus–Ig complexes, which decreased within 3 h, increased to a maximum level at 12 h and then declined slowly up to 24 h. This was similar to the change in kinetics of virus–Ig complexes observed in A/PR8 virus-immunized mice (Fig. 1e). There seemed to be no difference in virus number within the immune complexes after infection among these immunized mice (Fig. 1e), although the different levels of the A/PR8 virus cross-reactive Abs, which were higher in A/PR8, A/Yamagata and A/Guizhou-X virus-immunized mice, in decreasing order (Table 1), were detected. This may be due to the virus number within the immune complexes.

**Fig. 1.** Kinetics of infectious virus production (a, b), the total number of viral genome copies (c, d) and number of viral genome copies within virus–Ig complexes (e) after infection with A/PR8 virus (capable of producing $7.4 \times 10^6$ p.f.u. per mouse in MDCK cell culture) in the nasal secretions of naive mice and mice immunized 4 weeks previously with the A/PR8 (H1N1), A/Yamagata (H1N1), A/Guizhou-X (H3N2) or B/Ibaraki viruses. The kinetics of total numbers of genome copies after intranasal administration of UV-inactivated viruses (equivalent to the amount of virus capable of producing $7.4 \times 10^5$ p.f.u. per mouse) in naive and immunized mice was analysed as a control and the results are shown in (c). The number of virus particles within the immune complexes was examined for virus–IgA complexes (e). The nasal secretions at each time point after infection were pooled from each group of three mice. Each point represents the mean number of p.f.u. or genome copy number ± SD of triplicate samples from the pooled nasal secretions. NW, nasal wash.
reaching a maximum level in the presence of 1 ng to 1 μg Ab ml⁻¹, as described for the detection system. Thus, the early virus elimination observed in these experiments depended on the antigenic relatedness between the immunizing and challenging viruses and may have been the result of capture of infectious virus by A/PR8-reactive Abs shortly after infection. In addition, the increase in the level of immune complexes may have been involved in the decreasing numbers of infectious and total virus. These results suggested that the formation of virus–Ig complexes shortly after infection is involved in early virus elimination in immunized mice.

**DISCUSSION**

In the present study, the kinetics of total virus number, virus within virus–Ig complexes formed after infection with influenza A virus and infectious virus was investigated in nasal secretions of naive mice and mice previously immunized with different influenza A and B viruses. Total virus number or the virus number within the immune complex may have been involved in the decreasing numbers of infectious and total virus. These results suggested that the formation of virus–Ig complexes shortly after infection is involved in early virus elimination in immunized mice.

A large difference was also observed between the number of virus particles capable of producing infectious virus in culture and that capable of producing infectious virus following infection of naive and immunized mice. When naive and immunized mice were infected intranasally with the A/PR8 virus, which is capable of producing 7.4 × 10⁵ p.f.u. per mouse in culture, the capacity of the virus in nasal secretions to produce infectious virus after 1–3 h was reduced from 1 out of 61 to 1 out of 10⁴ to 10⁵ virus particles (Fig. 1c). This reduction was due to inhibitory factors in the respiratory tract mucosa that inhibit influenza virus infectivity. Factors that are similar to or identical to N-acetylmuramidic acid-containing receptors for HA molecules on the surface of viruses are present in human and animal respiratory tract secretions and are known to inhibit the initiation of viral infection (Cone, 1999; Shugars, 1999). Further studies to identify inhibitory factors in the nasal secretions of naive and immunized mice are under way.

Based on the present results, the process of virus clearance from the upper respiratory tract of mice after infection can be divided into four stages. In stage 1, which extends from 0 to 3 h after infection, both infectious and UV-inactivated viruses were eliminated rapidly in both the naive and the immunized mice (Fig. 1c and e). Total virus number decreased to 10⁴–10⁵ copies. This rapid decrease is likely to be the result of non-specific elimination of virus via physiological cleaning systems in the nose, such as the ciliary movement of the epithelial cells of the nasal mucosa.

### Table 1. Nasal anti-A/PR8 virus-specific and anti-A/PR8 HA-specific IgA and IgG titres and serum anti-A/PR8 virus neutralization titres in mice immunized 4 weeks earlier with various viruses

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Anti-A/PR8 virus (ng ml⁻¹)*</th>
<th>Anti-A/PR8 HA (ng ml⁻¹)</th>
<th>Neutralization†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IgA</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>A/PR8 (H1N1)</td>
<td>43.0 ± 14.6</td>
<td>9.2 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>A/Yamagata (H1N1)</td>
<td>12.5 ± 3.0</td>
<td>4.6 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>A/Guizhou (H3N2)</td>
<td>7.1 ± 4.8</td>
<td>5.2 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>B/Ibaraki</td>
<td>&lt;5.0</td>
<td>&lt;2.0</td>
<td></td>
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*Amount of antibody, assayed by ELISA.
†Serum dilution giving a 50% reduction in number of p.f.u. ND, Not detected.

### Table 2. Nasal anti-HA IgA and IgG titres in mice immunized 4 weeks earlier with various viruses

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Origin of purified HA used for ELISA</th>
<th>Anti-HA Ab titre (2n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgA</td>
</tr>
<tr>
<td>A/Yamagata (H1N1)</td>
<td>A/Yamagata</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>A/Guizhou (H3N2)</td>
<td>A/Guizhou</td>
<td>5.7 ± 1.3</td>
</tr>
<tr>
<td>B/Ibaraki</td>
<td>B/Ibaraki</td>
<td>4.3 ± 0.5</td>
</tr>
</tbody>
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At the same time, the number of infectious virus particles in immunized mice decreased from $7.4 \times 10^5$ to 10 p.f.u. ($ml$ nasal wash)$^{-1}$, which was lower than that observed in naive mice (Fig. 1a). The lower number of infectious virus particles in the immunized mice 3 h after infection was most likely the result of neutralization of infectious virus in the presence of virus-reactive Abs. The importance of neutralization in reducing infectious virus levels was reflected in the formation of immune complexes between challenge virus and existing Abs (Fig. 1e). Thus, it can be concluded that immune complexes are involved in the clearance of infectious virus in immunized mice. The discrepancy between the total virus number 0–12 h after challenge with infectious virus ($10^6$ copies) and that observed after administration of UV-inactivated virus ($10^3$ copies) (Fig. 1c) suggested that susceptible host cells were infected by a small number of viruses (Lamb & Krug, 2001).

In stage 2, which extends from 3 to 12 h after infection, the total virus number, infectious virus number and the virus number within the immune complexes increased after virus infection, although the total virus number decreased to its lowest level at 12 h (about $10^3$ copies) after administration of UV-inactivated virus (Fig. 1c). These results may be explained by an increase in the number of progeny virus released from infected epithelial cells and an increase in the number of immune complexes formed between progeny virus and the existing Abs. Progeny virus may be released from infected epithelial cells 3–5–6 h after infection and continue to be released over several hours until the infected epithelial cells die (Reinacher & Weiss, 1975; Lamb & Krug, 2001). The progeny virus must be captured by existing IgA Abs in nasal secretions, which are secreted across the epithelial cells of the nasal mucosa, and IgG Abs, released from the serum by diffusion (Mestecky & McGhee, 1987; Murphy, 1994). Infectious virus was produced when a high titre of virus ($7.4 \times 10^5$; Fig. 1), but not a low titre of virus ($7.4 \times 10^2$; data not shown), was used as the challenge infection dose in immunized mice. Thus, under the infection conditions with the high-titre virus, not all virus was neutralized by existing Abs, leading to infection of host cells. The degree of infectious virus development was lower in immunized mice than in naive mice, which had no pre-existing Abs (Fig. 1a). However, the difference in the total virus number between immunized and naive mice was not as marked as the difference in the number of infectious virus particles. This may be the result of free viral genome copies and viral genome copies within the immune complexes persisting in nasal secretions for the first 12 h after infection so that the contribution of existing Abs in reducing total virus numbers is not detected.

In stage 3, which extends from 12 h to 3 days after infection, total virus, infectious virus and virus within immune complexes all decreased slowly in number. The degree of reduction of total and infectious virus number correlated with the level of anti-A/PR8 virus-reactive Abs observed in A/PR8, A/Yamagata and A/Guizhou-X virus-immunized mice, in decreasing order (Table 1; Fig. 1). The immune mechanisms providing protection against influenza virus infection are redundant at this stage of infection (Couch & Kasel, 1983; Murphy & Clements, 1989; McMichael, 1994). It has been reported that influenza virus-specific CTL responses are involved in the clearance of viruses from the lung and nose and that the CTL responses appear 3 days after a secondary infection (Yap & Ada, 1978; Flynn et al., 1998; Wiley et al., 2001). This observation that CTL activity was detected only after the third day of secondary infection in the different subtype virus-immunized mice suggested that pre-existing Abs in the A/PR8 and the A/Yamagata virus-immunized mice were involved in the elimination of challenge viruses by forming virus-Ig complexes. This was shown to occur within 24 h and 3 days, respectively, in A/PR8 and the A/Yamagata virus-immunized mice based on estimations of the number of infectious virus particles following infection (Fig. 1; Table 1).

In stage 4, which extends from day 3 onwards after infection, the amount of infectious virus decreased slowly and disappeared within 4 days after A/PR8 virus infection of A/Guizhou-X virus-immunized mice. CTL memory cells are involved in the clearance of virus-infected cells in mice immunized with different subtype viruses (Wilson et al., 1987; Murphy & Clements, 1989; McMichael, 1994; Wiley et al., 2001). Thus, in mice immunized with different subtype viruses, secondary CTL responses accelerated by the challenge infection may be involved in virus clearance from day 3 after infection. Investigations are being carried out to define further the events in this stage.

**REFERENCES**


