Table 2 Primers and probes for quantitative real-time RT-PCR and *in situ* hybridization AT tailing

Target	*Primer and probe*	*Sequence*	*Gene bank or reference*
Influenza virus-matrix	Forward primer	5′-AGCAAAAGCAGGTAGATRTT-3′	CY006300
	Reverse primer	5′-TCGGCTTTGAGGGGG-3′	Ref. Ng *et al*[31]
	Probe	5′-FAM-AMCCGAGGTCGAAACGTAYG-TAMRA-3′	
TNF-α	Forward primer	5′-CAGAGGGAAGAGTTCCCCAGG-3′	NM_000594.2
	Reverse primer	5′-GGCTACAGGCTTGTCACTGG-3′	
	Probe	5′-FAM-TGGCCCAGGCAGTCAGATCATCTTCTCG-TAMRA-3′	
IL-6	Forward primer	5′-GAAGCTCTATCTCCCCTCCAGG-3′	NM_000600.3
	Reverse primer	5′-GCAACACCAGGAGCAGCC-3′	
	Probe	5′-FAM-ACTCCTTCTCCACAAGCGCCTTCGGT-TAMRA-3′	
IL-8	Forward primer	5′-CTTGGCAGCCTTCCTGATTTC-3′	NM_000584.2
	Reverse primer	5′-GCACTGACATCTAAGTTCTTTAGCA-3′	
	Probe	5′-FAM-GCTCTGTGTGAAGGTGCAGTTTTGCCA-TAMRA-3′	
RANTES	Forward primer	5′-GCATCTGCCTCCCCATATTCC-3′	NM_002985.2
	Reverse primer	5′-CCACTGGTGTAGAAATACTCCTTGA-3′	
	Probe	5′-FAM-CTGCTTTGCCTACATTGCCCGCCCA-TAMRA-3′	
IP-10	Forward primer	5′-GCCATTCTGATTTGCTGCCTTA-3′	NM_001565
	Reverse primer	5′-TGCAGGTACAGCGTACAGTTC-3′	
	Probe	5′-FAM-AGTGGCATTCAAGGAGTACCTCTCTCT-TAMRA-3′	
β-Actin	Forward primer	5′-TGAGCGCGGCTACAGCTT-3′	NM_00110
	Reverse primer	5′-TCCTTAATGTTCACGCACGATTT-3′	Ref. Krafft *et al*[32]
	Probe	5′-FAM-ACCACCACGGCCGAGCGG-TAMRA-3′	
H5N1 virus -NP	Sense probe	5′-GCAAGGGTCAACTCTCCCGAGGAGATCTGGAGCTGCTGGT-$(AT)_{10}$-3′	AY651530
	Antisense probe	5′-ACCAGCAGCTCCAGATCTCCTCGGGAGAGTTGACCCTTGC-$(AT)_{10}$-3′	
Rabies virus -NP	Antisense probe	5′-CAGTGGGGTCCCTTGTCAGCTCCATACCTCCCGTCAGAGC-$(AT)_{10}$-3′	AB573762.1

rabbit or anti-mouse IgG (Molecular Probes) and AlexaFluor 568-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes) were used as secondary antibodies. Nuclei were stained with TO-PRO-3, a specific nucleic acid stain (Molecular Probes). Confocal laser scanning microscopy was used to visualize double immunofluorescence staining as described previously.[18,23,27]

RNA Extraction

RNA was extracted from formalin-fixed, paraffin-embedded tissue sections (10μm × 3) using a Pure-Link FFPE total RNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Each sample was treated with DNase I to eliminate DNA contamination using a Turbo DNA-free kit (Ambion, Austin, TX, USA). The total RNA concentration was determined from spectrophotometric optical density measurements.

qRT-PCR Assays

Copy numbers for each RNA of interest and human β-actin mRNA from each sample were determined by qRT-PCR performed in a Mx3005P (Stratagene, La Jolla, CA, USA) using a QuantiTect Probe RT-PCR kit (Qiagen GmbH, Hilden, Germany). Human β-actin mRNA was used as an internal reference gene that provided a normalization factor for the amount of RNA isolated from a specimen. The copy numbers of H5N1 per cell were calculated using β-actin mRNA copy number, which was estimated to be 1500 copies per cell. For H5N1 RNA, we used a primer-probe set that amplified a segment within the matrix protein (M) region of H5N1 RNA. The RT-PCR thermal cycling conditions were 50 °C for 30 min, then 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, and 60 °C for 1 min. All samples were run in triplicate. Primers and probes were synthesized by Sigma Genosys (Sigma-Aldrich, St Louis, MO, USA; Table 2).

Results

Patients

The characteristics of the five patients are presented in Table 1. H5N1 infection was confirmed by detecting H5N1 RNA in bronchial aspirate by RT-PCR. The durations of illness were 8, 10, 13, 16 and 18 days for cases 1, 2, 3, 4 and 5, respectively. The patients did not have significant medical histories or underlying diseases. All of them had a chance to be infected because of direct contact with sick poultry. Clinical symptoms exhibited by all patients included fever, cough and dyspnea. The average day of admission, which corresponded to when patients

complained of dyspnea, was day 8 of the illness. Soon after admission, they were placed on a ventilator because of reduced oxygen saturation. Laboratory data on admission showed that white blood cell count was low for all cases (1700–3400/mm^3). Antibiotics had been prescribed in all cases before admission. Antiviral treatment with oseltamivir was used in cases 2, 3 and 4 after admission. Methylpredonisolone was administered in all cases except for case 5. *Hemophilus influenza* was cultured from the sputa of case 5 on admission.

Histopathological Findings in the Lung

Acute intra-alveolar edema, congestion and/or hemorrhage, desquamation of pneumocytes, interstitial and intra-alveolar inflammatory cell infiltration, fibrosis and type II pneumocyte hyperplasia were observed. Three cases died before day 13 of the illness and presented with the exudative phase of diffuse alveolar damage (Figures 1a and c). The other two cases died after day 16 of the illness and presented with the proliferative phase of diffuse

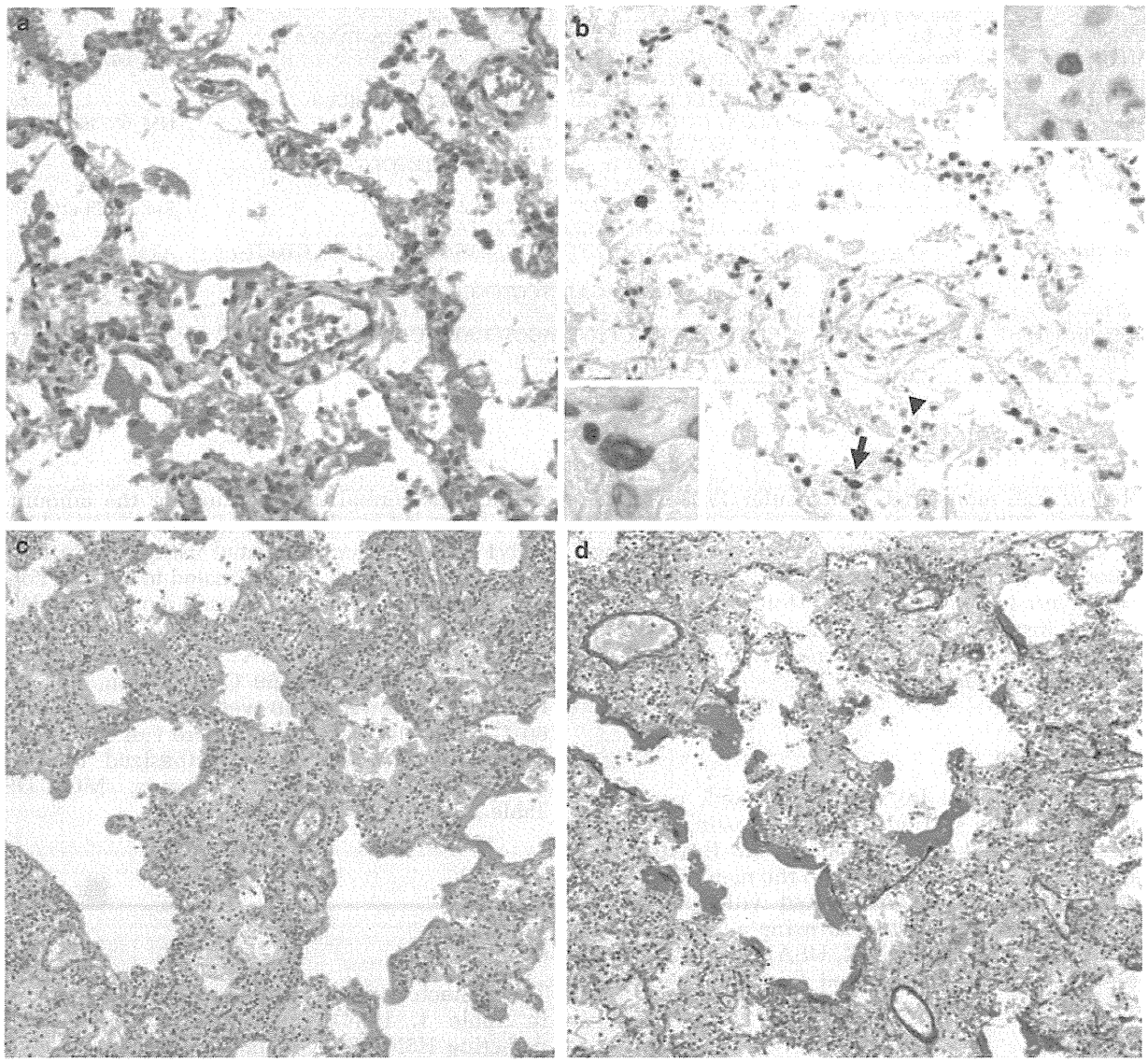

Figure 1 Representative histopathological findings in lung sections following hematoxylin–eosin (HE) (**a**, **c**, **e**) or Elastica–Masson Goldner staining (**d**). Immunohistochemistry was conducted to detect type A influenza virus nucleoprotein antigen (InfA-NP) (**b**). *In situ* hybridization AT-tailing (ISH-AT) (**f–h**) was also used. (**a**) Exudative phase of diffuse alveolar damage in Case 1. (**b**) InfA-NP antigen (brown) was detected in the nucleus (arrow head, upper inset) or cytoplasm (arrow, lower inset) of alveolar epithelial cells for Case 1. (**c**) Exudative phase of diffuse alveolar damage in case 3. (**d**) Elastica–Masson Goldner staining showed hyaline membrane formation (red) for case 3. (**e**) Proliferative phase of diffuse alveolar damage. The numbers of fibroblasts and myofibroblasts were increased in the alveolar septa and alveolar space in case 4. (**f**) ISH-AT using an antisense H5N1 nucleoprotein (NP) probe was able to detect H5N1 mRNA. (**g**) ISH-AT with a sense H5N1 NP probe detected H5N1 genomic RNA. (**h**) ISH-AT using an antisense rabies virus nucleocapsid probe as a negative control. Original magnifications, ×10 (**c**, **d**), ×20 (**a**, **b**), ×40 (**b**, inset, **f–h**).

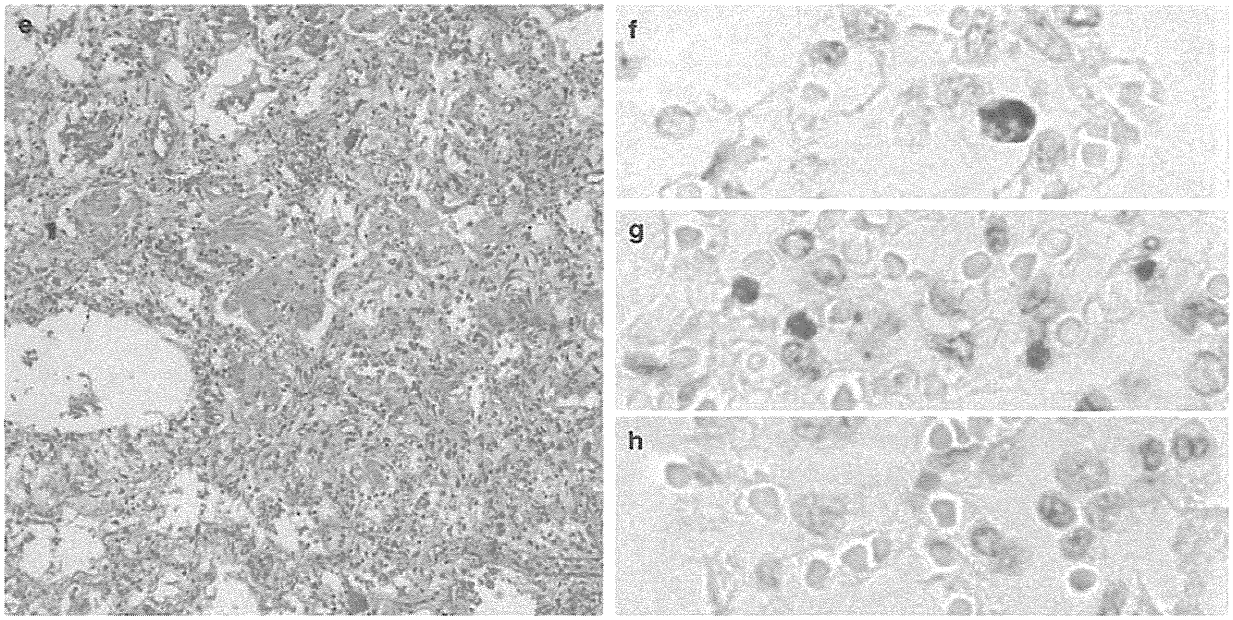

Figure 1 Continued.

alveolar damage. The numbers of myofibroblasts and fibroblasts were increased in case 4 (Figure 1e). Hyaline membrane formation was shown by Elastica–Masson Goldner staining (Figure 1d).

Histopathological Findings in Extrapulmonary Organs

Several extrapulmonary organs were examined in cases 2 and 3 (Table 1). Inflammatory cell infiltration was not observed in any sections from extrapulmonary tissues. In the liver, we observed focal mild fatty changes around a lobule with a ballooning change of hepatocytes in both cases. Reactive hemophagocytosis[3,5,6] and lymphoid depletion,[5,17] which are sometimes associated with hypercytokinemea, were not remarkable in the spleen. Renal tubular necrosis and congestion in the kidney[17] were not observed. The pathological findings in extrapulmonary organs from both cases were limited and appeared to be caused by hypoxic changes rather than H5N1 infection.

Characterization of Infiltrating Cells

The infiltrating cells in the alveolar septa and alveolar space (Figure 2a) were characterized using immunohistochemistry on serial lung sections from the exudative phase of diffuse alveolar damage. Most infiltrating cells were MPO-positive (Figure 2b) and/or CD68 clone KP-1-positive (Figure 2c). MPO was expressed in neutrophils, monocytes and their precursors. CD68 clone KP-1 was expressed in not only monocytes/macrophages but also myeloid precursors and neutrophils. Neutrophil elastase was expressed in some of the infiltrated cells, suggesting they were neutrophils or precursor cells (Figure 2d). CD68 clone PGM-1 is more specific for mature macrophages in comparison with CD68 clone KP-1. CD68 clone PGM-1-positive alveolar macrophages were also detected (Figure 2e). Taken together, the results indicate that the infiltrating cells were mainly neutrophils and monocytes/macrophages, and their precursors. CD8-positive cytotoxic T lymphocytes were rarely detected in this region (Figure 2f). Several CD8-positive T lymphocytes were detected in other areas of the same lung section, but there were few of these in total.

Viral Load

The relative levels of H5N1 viral RNA (genomic RNA and mRNA) in formalin-fixed, paraffin-embedded sections were quantified by qRT-PCR using primer-probe sets for the H5N1 M protein and β-actin gene sequences (Table 2). The relative H5N1 viral copy number per cell was calculated as described in the Materials and methods section. H5N1 RNA was under the detection limit (UDL) in all extrapulmonary tissue sections. The viral load varied among the lung regions from the same case (Figure 4a). The highest copy number of H5N1 RNA was seen from the lung in case 1, who had the shortest duration of illness. The lung in the proliferative phase of diffuse alveolar damage (cases 4 and 5) presented with a low viral load. In case 5, who died on day 18 of the disease, the amount of viral RNA was UDL in all lung sections (Figure 4a).

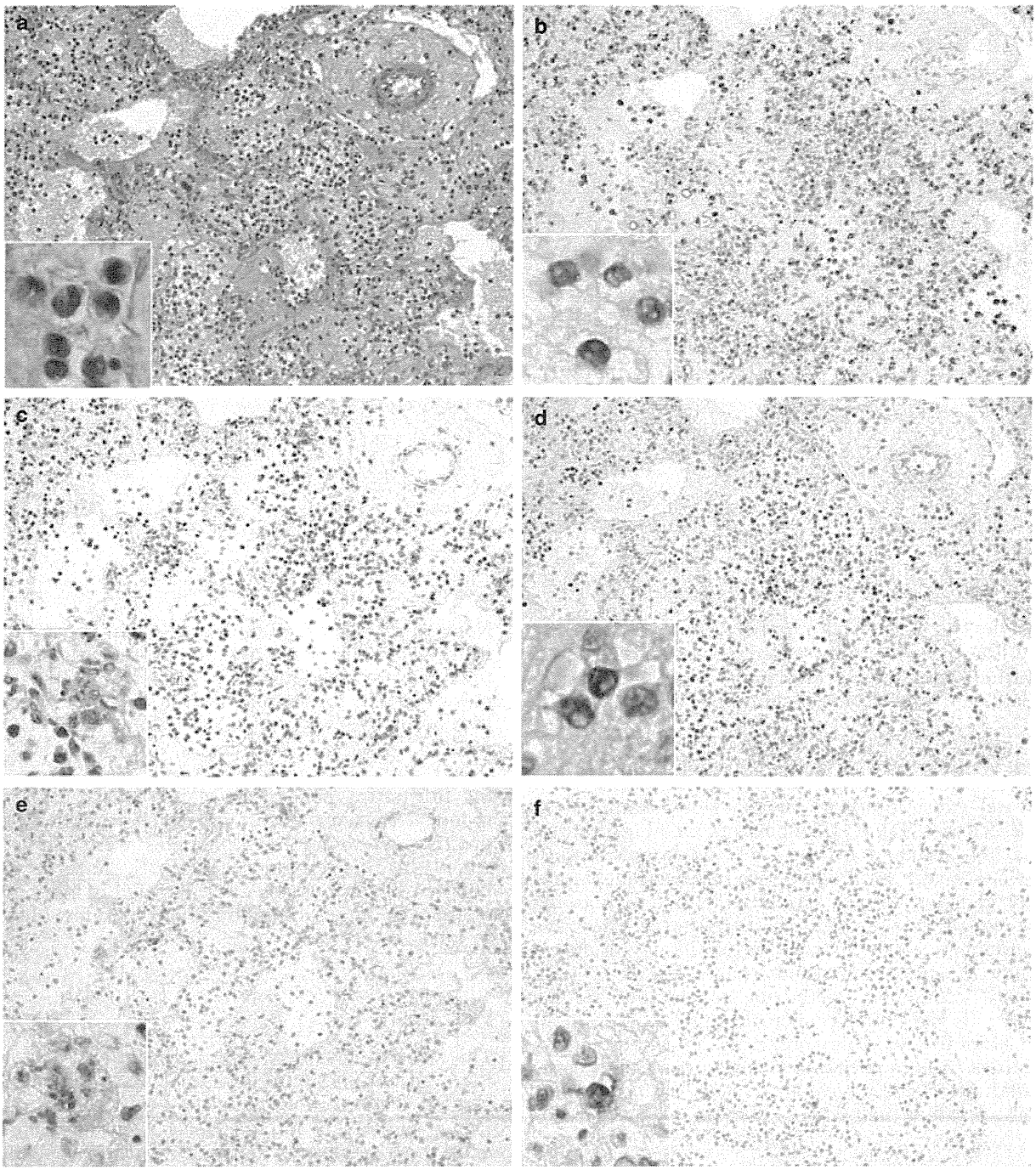

Figure 2 Hematoxylin–eosin (HE) staining (**a**) and immunohistochemistry for cell type-specific marker proteins (**b–f**) in serial lung sections from case 3. (**a**) Inflammatory cells infiltrated the alveolar septa and alveolar spaces. The phenotype of infiltrating cells were characterized using immunostaining for the detection of myeloperoxidase (MPO) (**b**), CD68 clone KP-1 (**c**), neutrophil elastase (**d**), CD68 clone PGM-1 (**e**) and CD8 T cells (**f**). Positive signals are indicated by brown staining. Original magnification, × 20 (**a–f**), × 40 (**a–f**, inset).

H5N1-Infected Cells Detected by Immunohistochemistry and *In Situ* Hybridization

The distribution of InfA-NP was examined by immunohistochemistry using monoclonal antibodies against the protein. It was detected in the lung tissue sections from cases 1, 2 and 3 (Table 1, Figure 1b). H5N1 mRNA and genomic RNA were detected separately in the lung sections of cases 1, 2 and 3 using ISH-AT with anti-sense and sense probes (Figures 1f and g). The detection of influenza mRNA was an indicator of virus proliferation in the

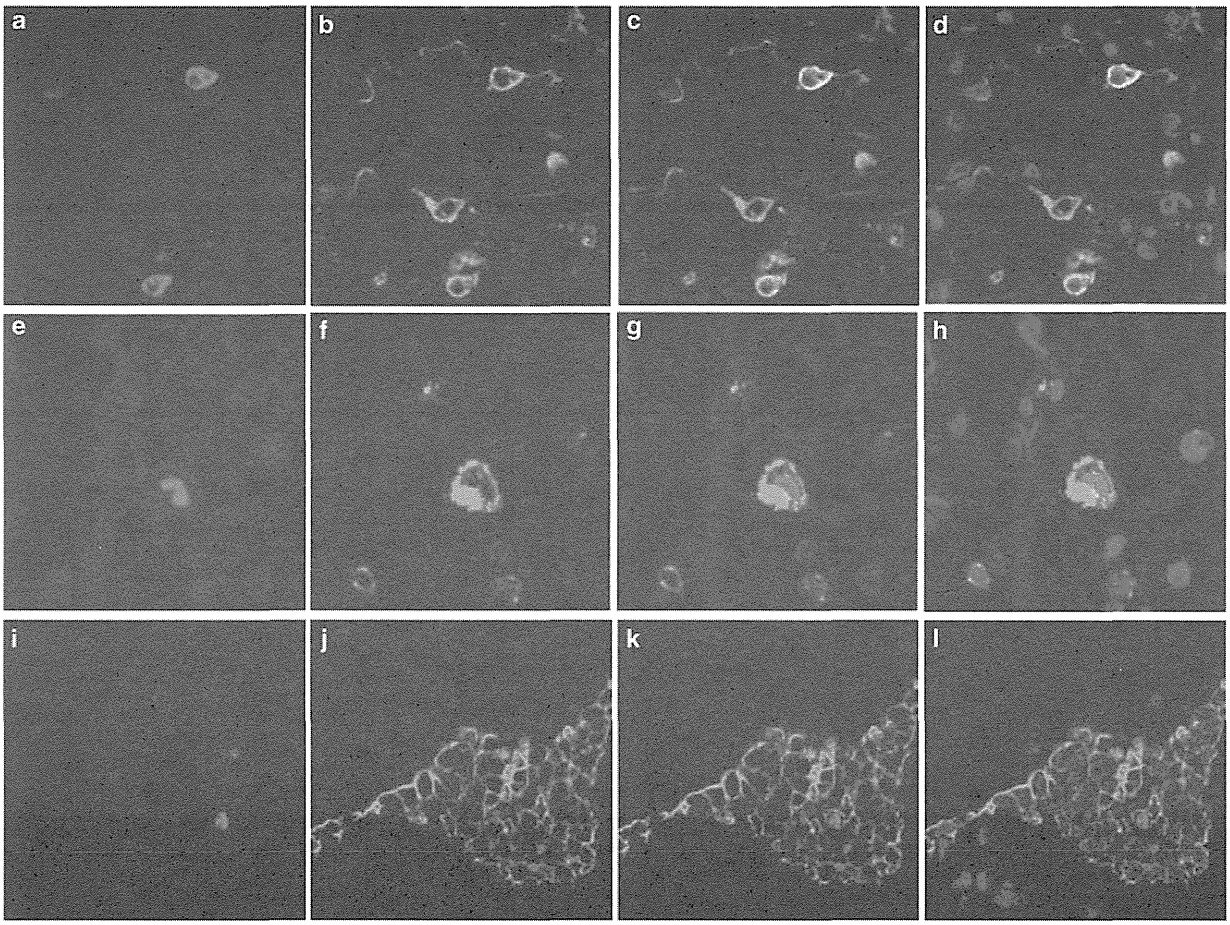

Figure 3 Phenotype of influenza A nucleoprotein antigen (InfA-NP)-positive cells. (**a**, **e**, **i**) InfA-NP immunoreactivity can be seen in red. (**b**) Immunoreactivity for surfactant apoprotein D (SP-D) in type II pneumocytes. (**f**) CD68 clone KP-1 in monocytes. (**j**) Cytokeratin (AE1/AE3) in bronchiolar epithelial cells. (**c**, **g**, **k**) Colocalization for each type is also presented. (**d**, **h**, **l**) TO-PRO-3 nucleic acid staining (blue) revealed the InfA-NP in nuclei. Original magnification, ×400.

lung. The InfA-NP-positive cells (red) were identified using double immunofluorescence staining for cell type-specific marker proteins (green) and TO-PRO-3 nucleic acid staining (blue). Positive signals were visualized by confocal laser scanning microscopy (Figure 3). The InfA-NP signals (Figures 3a, e and i) were mainly detected in the SP-D-positive type II pneumocytes (Figure 3b), and the CD68-positive monocytes/macrophages (Figure 3f). They were also detected in AE1/AE3-positive bronchiolar epithelial cells (Figure 3j). InfA-NP signals were detected in the nuclei (Figures 3d, h, and i).

Cytokines and Chemokines

The elevation of cytokine and chemokine levels occurred in H5N1-infected lungs. Their expression levels were examined by quantifying the mRNA copy number of TNF-α, IL-6, IL8, RANTES and IP-10 in the five cases. The extracted RNA from 3 to 5 lung regions from each case was analyzed separately. The expression levels were variable from every region of the same case. Case 1 presented with the highest titers of cytokines and chemokines of all. In case 5, only IL-8 mRNA was detected. The expression level of every cytokine and chemokine correlated with the copy number of H5N1 RNA (Figure 4g). This suggested that the local elevation of cytokines and chemokines in the lung were possibly caused by H5N1 infection in the same region.

Next, we tried to detect these cytokines and chemokines in the lungs of cases 1 and 3 using immunohistochemistry (Figure 5). TNF-α, IL-6, IL-8, RANTES and IP-10 were detected at much higher levels in case 1 compared with case 3, which was compatible with the copy number of each mRNA shown in Figure 4g. The phenotype of each cytokine/chemokine-positive cell was determined using double immunofluorescence staining. Cells expressing RANTES could not be identified. TNF-α was detected in a MPO-positive monocytes (Figure 6a-3) and SPD-positive type II pneumocytes (Figure 6b-3). IL-6 was detected in PGM-1-positive

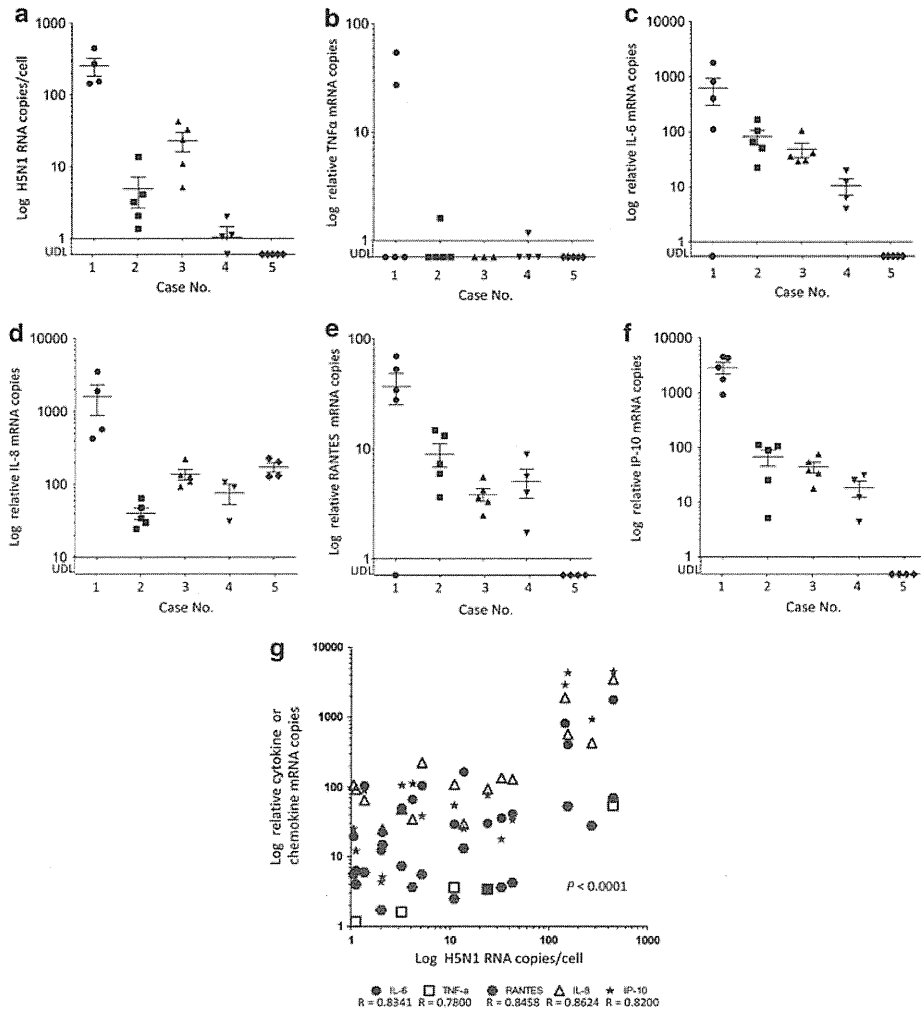

Figure 4 H5N1 RNA and proinflammatory cytokine or chemokine expression levels in several lung regions. The x axis indicates the case number. The durations of disease for cases 1, 2, 3, 4 and 5 were 8, 10, 13, 16 and 18 days, respectively. The y axis indicates H5N1 RNA (**a**), tumor necrosis factor-alpha (TNF-α) (**b**), interleukin (IL)-6 (**c**), IL-8 (**d**), regulated on activation normal T-cell expressed and secreted (RANTES) (**e**) and interferon-gamma-inducible protein of 10 kDa (IP-10) (**f**) mRNA copy numbers on a logarithmic scale. ●, case 1; ■, case 2; ▲, case 3; ▼, case 4; and ◆, case 5. (**g**) Correlation between copy numbers of H5N1 RNA and cytokine or chemokine mRNA in lung tissue. Data from the five cases (**a–f**) were combined, with any values below the limit of detection (UDL) excluded. The horizontal lines indicate the mean, and vertical error bars indicate the mean ± s.d., $P<0.0001$.

monocytes/macrophages (Figure 6c-3) but also in an EMA-positive alveolar epithelial cells (Figure 6d-3) and in CD34-positive endothelial cells (Figure 6e-3). IL-8 was detected in PGM-1-positive monocytes/macrophages (Figure 6f-3). IP-10 was detected in PGM-1-positive monocytes/macrophages (Figure 6g-3) and EMA-positive bronchiolar epithelial cells (Figure 6h-3).

Discussion

All post-mortem biopsied or autopsied cases with H5N1 infection reported to date have shown acute respiratory distress syndrome clinically and diffuse alveolar damage in lung histopathology.[3–18] The five cases analyzed in this study also suffered from viral pneumonia, which led to acute respiratory distress syndrome. H5N1 antigens and RNA were detected in pneumocytes and monocytes/macrophages in cases 1, 2 and 3 (Figures 1e–g). In particular, these three cases presented with the exudative phase of diffuse alveolar damage *via* histopathology, with infiltration of inflammatory cells in the alveolar septa and alveolar space remarkably high. Immunohistochemistry revealed that the inflammatory cells were not lymphocytes, but mostly MPO- and/or CD68 clone KP-1-positive neutrophils or monocytes/macrophages (Figure 2). A more detailed study is necessary to elucidate the mechanism of infiltration for these cells in the H5N1-infected lung.

H5N1 patients presented with dysregulation of cytokine and chemokine levels, which is often referred to as a 'cytokine storm'. This is thought to

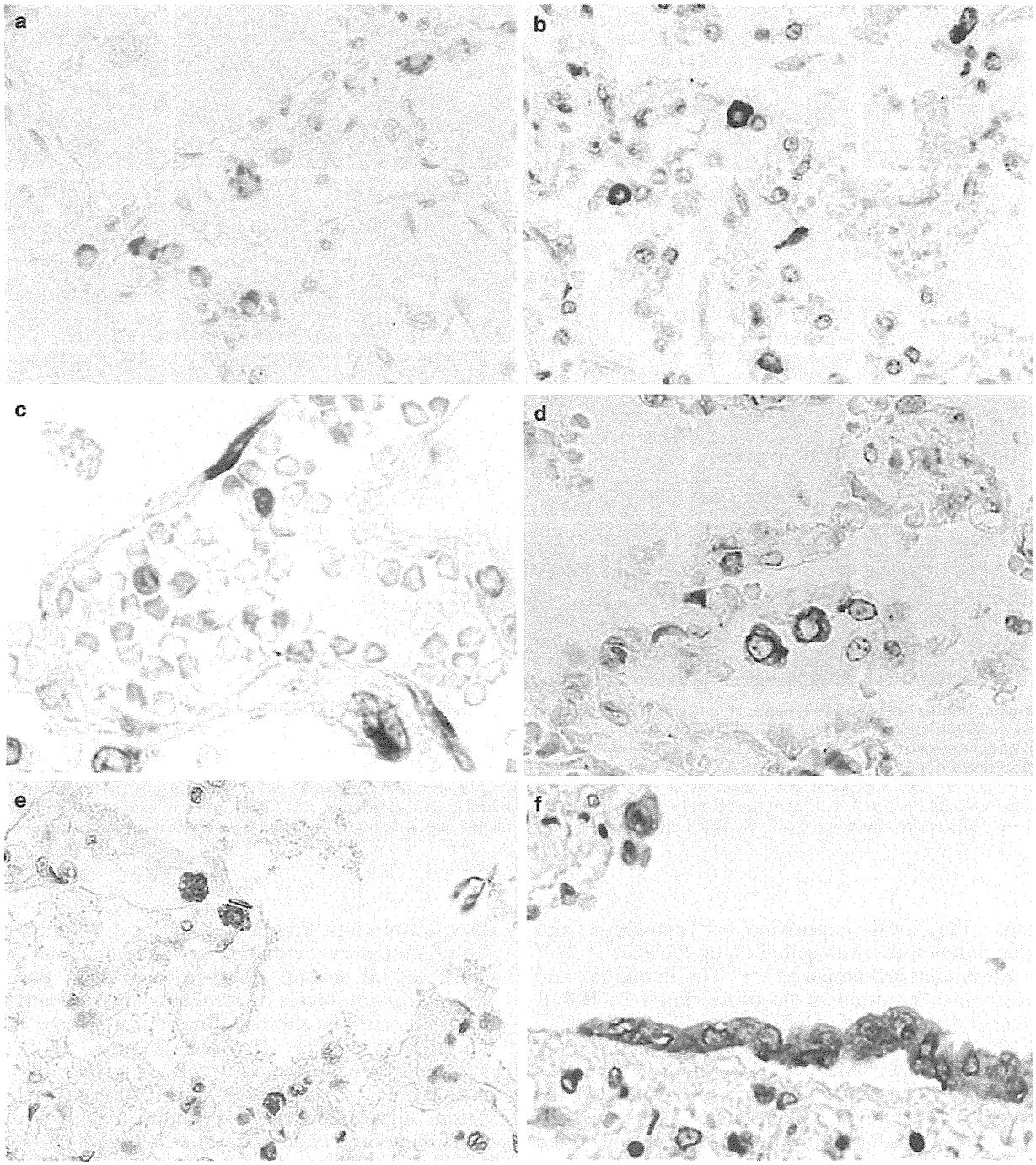

Figure 5 Immunohistochemistry on lung tissue sections for the detection of cytokines and chemokines. (**a**) Tumor necrosis factor-alpha (TNF-α), (**b**, **c**) interleukin (IL)-6, (**d**) IL-8, (**e**) regulated on activation normal T-cell expressed and secreted (RANTES) and (**f**) interferon-gamma-inducible protein of 10 kDa (IP-10) were detected in the cytoplasm of several cells. (**c**) IL-6 immunoreactivity was observed in endothelial cells. (**f**) IP-10 immunoreactivity was seen in bronchiolar epithelial cells. A positive signal is indicated by brown staining. Original magnification, × 40.

be one of the key mechanisms in the pathogenesis of H5N1 infection.[3,33] According to *in vitro* experiments, H5N1 infection of primary human macrophages, along with alveolar and bronchial epithelial cells induced proinflammatory cytokines and chemokines more potently than seasonal influenza virus infection.[34–36] Animal experiments also showed induction of proinflammatory cytokines and chemokines occurred because of H5N1 infection.[37,38] In H5N1-infected human

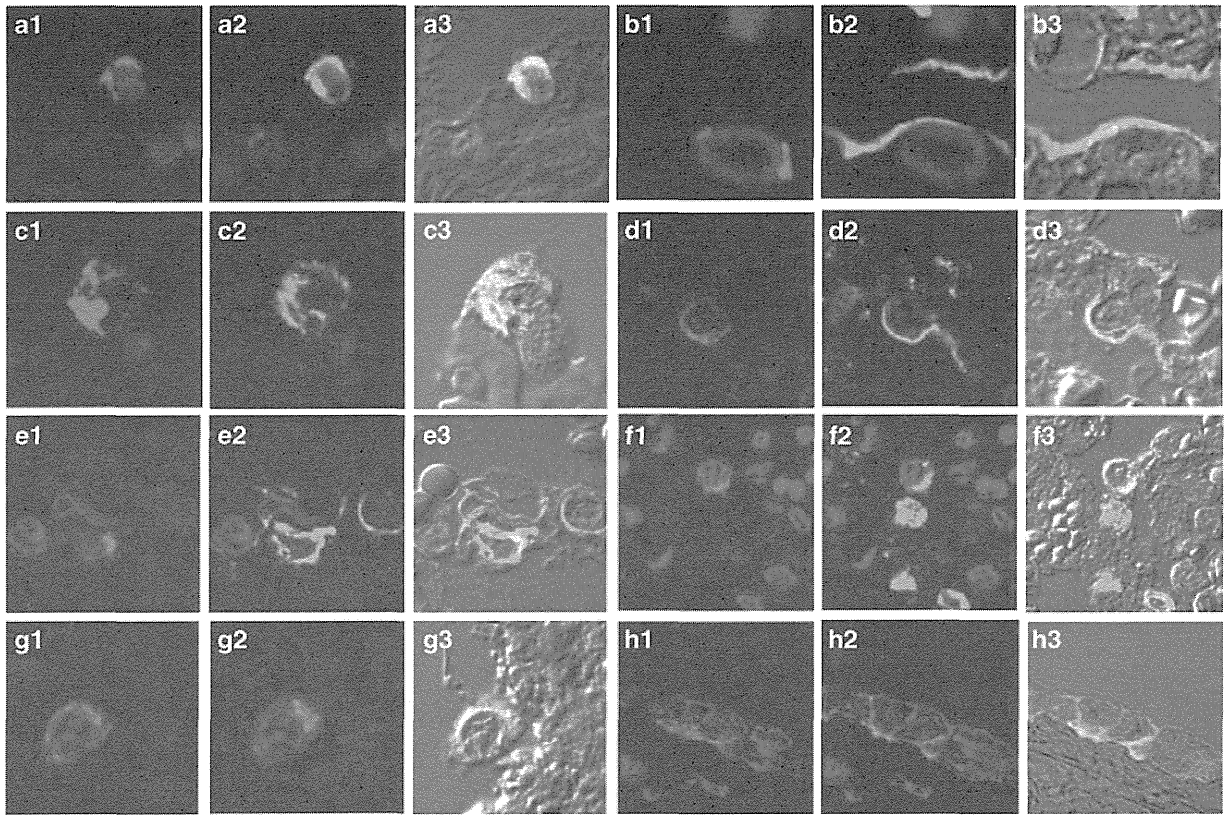

Figure 6 Double immunofluorescence staining for phenotype determination of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6-, IL-8- or IP-10-positive cells. (**a-1**, **b-1**) TNF-α, (**c-1**, **d-1**, **e-1**) IL-6, (**f-1**) IL-8 and (**g-1**, **h-1**) interferon-gamma-inducible protein of 10 kDa (IP-10) immunoreactivity (red) and cell type-specific marker protein immunoreactivity (green). (**a-3**, **b-3**, **c-3**, **d-3**, **e-3**, **f-3**, **g-3**, **h-3**) Colocalization of immunostaining. (**a-2**) Myeloperoxidase (MPO) in monocytes. (**b-2**) Surfactant apoprotein D (SP-D) in type II pneumocytes. (**c-2**, **f-2**, **g-2**) CD68 clone PGM-1 in monocytes/macrophages. (**d-2**) Epithelial membrane antigen (EMA) in alveolar epithelial cells. (**e-2**) CD34 in endothelial cells. (**h-2**) EMA in bronchiolar epithelial cells. TO-PRO-3 nucleic acid staining (blue) is shown. Differential interference contrast (DIC) images are shown in **a-3**, **b-3**, **c-3**, **d-3**, **e-3**, **f-3**, **g-3** and **h-3**. Original magnification, ×600.

lungs, the local expression of cytokines and chemokines was investigated using RT-PCR[9–11,13,17] or immunohistochemistry.[4,15,16] The cytokines and chemokines reported to be upregulated in H5N1-infected lungs were: TNF-α,[4,9,10,13,16,17] IFN-α/β,[11] IP-10,[11,13,16,17] RANTES,[16,17] MIP-3β,[17] IL-6,[16] IFN-γ,[16] IFN-β,[16] IL-8,[16] MCP-1,[16] and MIP-1α.[15,16] In this study, based on these findings, we quantified the expression of five proinflammatory cytokines and chemokines in formalin-fixed, paraffin-embedded H5N1-infected lung tissues. We examined 3–5 lung regions per case. It was impossible to examine the time course of expression for each mediator in the post-mortem biopsied or autopsied lungs. The five patients in this study had no significant medical histories or underlying diseases, which could possibly affect expression levels of cytokines and chemokines. Furthermore, all formalin-fixed, paraffin-embedded samples were prepared using the same procedures at the same hospital. We have presented the expression levels of each mediator in the lungs of the five cases in order of shortness of disease duration (Figure 4). The local induction of proinflammatory cytokines and chemokines were confirmed in several H5N1-infected lung tissues. The expression levels of cytokines and chemokines in case 1, with the shortest duration of disease, were the highest among all cases (Figures 4b–f). In addition, the expression levels of cytokines and chemokines correlated with viral load in every lung region, suggesting that H5N1-induced upregulation of cytokines and chemokines in the lung (Figure 4g). Double immunofluorescence staining revealed that cells expressing the cytokines and chemokines were mainly monocytes/macrophages or epithelial cells (Figure 6). Our results were consistent with those previously reported.[4,16] We also noticed the new finding that IL-6 was expressed in EMA-positive alveolar epithelial cells (Figure 6d-3), CD34-positive endothelial cells (Figure 6e-3), and PGM-1-positive monocytes/macrophages (Figure 6c-3). It should be noted that IL-6 was also detected in endothelial cells, which may be related to local vascular injury in H5N1-infected lung (Figure 6e-3).

The most important characteristics of H5N1 infection that distinguish it from other subtypes of influenza virus infection, are that H5N1 disseminates beyond the respiratory system.[14] For disseminated infection, H5N1 should have been in the bloodstream at some point. Virus isolation from peripheral blood is considered evidence of viremia.[33,39] Actually, virus antigens and H5N1 RNA have been reported to be detected in extrapulmonary tissues from several fatal cases.[6,9,10,12–15,17] The positive strand of H5N1 mRNA, indicative of viral replication, was detected by strand-specific RT-PCR in the intestines,[6,13,14] liver,[9,13] heart,[13,14] lymph node,[13] placenta[14] and brain.[14] On the other hand, histopathological findings were mostly nonspecific for H5N1 infection, such as hemophagocytotic activity, depletion of lymphoid cells, acute tubular necrosis, fatty changes in the liver and brain edema. More specific pathological changes, such as inflammatory cell infiltrations associated with the detection of viral antigen would be needed to show disseminate H5N1 infection. In extrapulmonary organs of two autopsied cases in this study, we were unable to obtain evidence of H5N1 dissemination. For both cases, illness lasted >10 days and the titers of H5N1 RNA were low, even in lung sections (Table 1, Figure 4a). Therefore, it might be reasonable to suggest that H5N1 RNA in extrapulmonary organs were below the level of detection. In addition, the histopathological findings of the extrapulmonary tissues were limited to nonspecific ischemic changes. The inconsistent results regarding the extent of H5N1 distribution in fatal cases are likely due to several factors, including the conditions of samples, the duration of disease and the medication given to patients. In addition to this, the differences in permissivity and immunological reactivity to H5N1 among patients should be also considered.

We investigated formalin-fixed, paraffin-embedded tissues from five fatal H5N1 cases. H5N1 viral load was highest in the lung of the case with the shortest duration of disease. Proinflammatory cytokine and chemokine mRNA copy numbers correlated with H5N1 RNA copy numbers in each lung region. In H5N1-infected lungs, monocytes/macrophages, epithelial cells and endothelial cells produced several cytokines and chemokines. We were unable to determine any dissemination of H5N1 beyond the respiratory organs in two autopsied cases. Further investigation is necessary to elucidate the pathogenesis of H5N1 infection in humans.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Review

Mucosal IgA responses in influenza virus infections; thoughts for vaccine design

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ABSTRACT

The current challenge in influenza vaccine design is to induce long-lasting protection not only against the vaccine strain, but also against drifted (point mutations in the surface antigens HA or NA) and even shifted (exchange of genome segments) strains. Several immune mediators that can induce cross-protection have been described, such as CD4 T-cells, CD8 T-cells and antibodies, including IgA. However, most vaccines are now administered intramuscularly or subcutaneously and subsequently relatively little is known on the role of local, mucosal responses. Since local IgA responses have been shown to play an important role in responses to natural infection, and IgA responses in mice were shown to also be involved in cross-protection, the research on mucosal influenza vaccines is currently expanding. However, the functioning of the mucosal immune system, especially in the respiratory tract, is just beginning to be revealed. Here, the current knowledge on the induction of IgA, the role of influenza specific IgA producing B-cells in anti-influenza immunity as well as the role of humoral memory responses induced upon vaccination will be reviewed.

Contents

1. Introduction

Seasonal influenza A virus infections cause millions of cases each year with the highest risk of complications in very young and very old people as well as immunocompromised patients, all lacking a strong immune response. In addition, also more infectious or pathogenic strains can infect people, such as the 2009 pandemic influenza A virus (A(H1N1)pdm09), or highly pathogenic avian influenza A H5N1 virus, respectively. In contrast to seasonal influenza viruses, highly pathogenic strains can be more threatening for young, healthy people in whom tissue damage can be the result of overly powerful host inflammatory responses [1].

In the case of both seasonal and newly evolved strains, the most efficient way to fight the disease is preventing it by means of vaccination. However, current influenza vaccines are effective against a single type of influenza only, thus for the seasonal vaccine necessitating the presence of multiple strains, as well as a yearly renewal of the vaccine. In addition to the possibility of a mismatch of the vaccine with the actual circulating influenza strains, newly evolved strains, such as H5N1 and A(H1N1)pdm09, highlighted the need for improved cross-protection. Ideally, a universal influenza vaccine would be developed, that induces a strong and long lasting memory response which is cross-protective to drift variants as well as across subtypes of the influenza virus (Fig. 1). Many factors are

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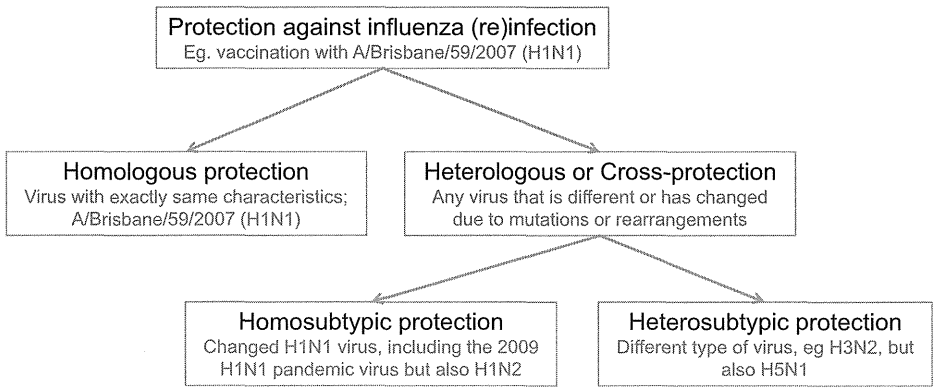

Fig. 1. Overview of different terms related to different types of protection. Since no official definition is available for these terms, this figure shows how these terms are used in this review. The definitions are based on the type of haemagglutinin antigen that the virus contains. For each term some virus strains that will fit the term are given in grey, based on an example of protection against A/Brisbane/59/2007 (H1N1).

known to play a role in cross-protection, but in this review we will focus on the current knowledge on the role IgA could play in realising universal protection. Importantly, rational design of IgA inducing vaccines has so far been hampered by a lack of knowledge: since local, tissue-specific, immune responses, including IgA, are often not measured [2], relatively little information is available. Consequently, the importance of the presence of IgA as well as the mechanisms via which IgA responses are induced and maintained are just beginning to be revealed [3].

2. The role of IgA in (cross-)protection from influenza infection

Pre-existing secretory IgA (S-IgA) antibodies can provide immediate immunity via their unique capability to eliminate a pathogen before it even passes the mucosal barrier and enters the human body [4], also termed immune exclusion [5]. Furthermore, IgA has also been shown to be very effective at disarming viruses in virus-infected secretory epithelial cells and in redirecting antigens to the lumen when they enter the lamina propria [3] (Fig. 2). These responses are all non-inflammatory, since IgA, unlike IgG, does not fix complement and thus does not activate the inflammatory complement pathway [6]. Therefore, a strong IgA response could be particularly important in case of highly pathogenic strains, where most complications are caused by uncontrolled pro-inflammatory responses.

Although the roles of S-IgA and serum antibodies are difficult to investigate independently, infection models in knockout mice showed that S-IgA normally does play an important role in protection against influenza [7,8]. Moreover, transfer of S-IgA from respiratory tract washings from immunized to naïve mice was shown to provide protection to challenge with a homologous or drifted strain [9] and several studies in mice showed induction of strong homosubtypic as well as modest heterosubtypic cross-protective IgA antibodies (Table 1).

Thus, IgA contributes to, but is not essential for the establishment of cross-protection to influenza. Interestingly, all mediators of cross-protection, such as CD8 T-cells [27], CD4 T-cells [28] or B-cells [29] seem to be partially redundant, since high degrees of protection were also observed in mice lacking CD4 T-cells, CD8 T-cells or B-cells [21,30,31].

3. IgA production

Antigen specific antibody producing B-cells can develop at two different types of locations, extrafollicular and in germinal centres (GC), and in a T-cell dependent or independent manner [32]. GC function as a specialized environment to support affinity maturation mediated by activation-induced deaminase (AID) induced somatic hypermutation [33]. In addition AID is involved in the production of the preferred antibody class, by influencing class switch recombination (CSR) of the heavy chain [33,34]. Most IgA memory B-cells (B_{Mem}) and long-lived IgA plasma cells develop in the GC of peripheral lymphoid organs and require T-cell help via

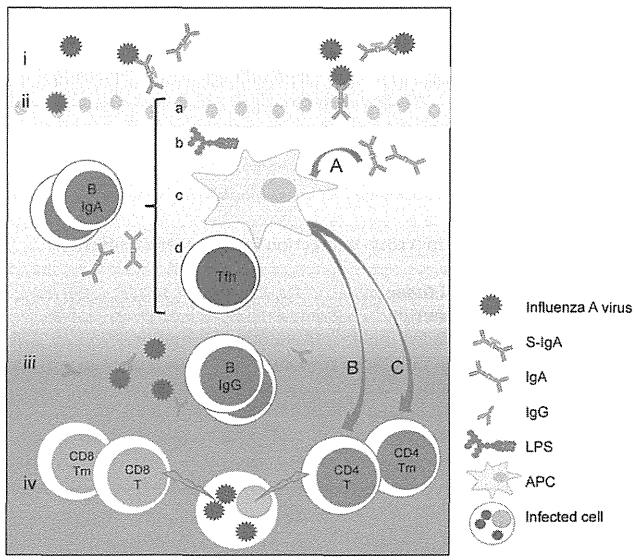

Fig. 2. The role of IgA in protection from influenza infection; function, induction and interaction. Function (i–iv): IgA can neutralize influenza viruses at the mucosal interface (i), even before they actually enter the host by crossing the mucosal barrier, thus existing IgA is the first line of defence upon reinfection or infection after vaccination. In addition, IgA can effectively clear the virus in infected epithelial cells (ii). Virus that could not be destroyed by IgA and successfully entered the host can be eliminated by IgG (iii), which is the main protection on which seasonal vaccination is based, and (iv) cytotoxic CD8 T-cells target host cells that were infected. CD4 cells can also kill infected cells, but are mostly involved in helping B- and CD8 T-cells to eliminate the influenza virus. Induction (a–d): induction of IgA (by influencing for example class switching or proliferation of plasmablasts) is influenced by tissue signals, for example from epithelial cells (a), innate signals, like viral or commensal products such as LPS that activate innate receptors (b), by different types of APC (c) and by helper T-cells, mostly Tfh (d). Interaction (A–C): IgA antibodies were found to influence the function of antigen presenting cells (APC) (A) and so indirectly have a share in the activation of effector T-cells (B) as well as activation of memory responses (C). Tfh: follicular helper T-cell, Tm: memory T-cell, APC: antigen presenting cell, S-IgA: secretory IgA.

Table 1
IgA mediated cross-reactivity to influenza A viruses in a mouse model.

Vaccine type	Adjuvant	Vaccine strain(s)	Cross-responsive strain(s)		Ref.
			Homo-subtypic	Hetero subtypic	
Only homosubtypic					
HA	CTB	H1N1	H1N1		[7]
HA	CTB + 0.2% holotoxin	H1N1	H1N1		[10]
HA	LTB + LT	H1N1	H1N1		[11]
HA	CTB	H1N1, H3N2	H1N1, H3N2		[12]
HA	CTB	H3N2	H3N2		[13]
Homosubtypic and heterosubtypic					
HA	Surf clam microparticles	H1N1 H3N2	H1N1	H1N1	[14]
HA	PolyI:C	H1N1 H3N2	H1N1	H1N1	[15]
HA	Chitin microparticles	H1N1 H3N2	H1N1	H1N1	[16]
HA	Mutant CT	H1N1 H3N2	H1N1	H1N1	[17]
HA	CTB	H1N1 H3N2	H1N1 H3N2	H3N2 H1N1	[9]
HA	CTB	H1N1	H1N1	H3N2	[18]
HA	CTB	H1N1 H3N2	H1N1	H1N1	[19]
HA	CTB	H1N1 H3N2	H1N1	H1N1	[20]
WIV	CT	H1N1	H1N1	H3N2	[21]
Only heterosubtypic					
HA	PolyI:$C_{12}U$	H1N1, H3N2		H5N1	[22]
HA	PolyI:$C_{12}U$	H1N1, H3N2		H5N1	[23]
LAIV and IIV[a]	–	H2N2		H5N1	[24]
WIV (formalin)[b]	–	H1N1, H1N2, H2N2, H3N1, H3N2, H5N4, H9N2		H5N1	[25]
WIV (formalin)	+/−LT	H3N2		H5N1	[26]

Only articles are included in which cross-protective IgA antibodies were demonstrated to be present. Vaccines were administered intranasally.
[a] Inactivated influenza virus, different from WIV.
[b] Ether split vaccine did not induce heterosubtypic protection.

CD40L (CD154) and TGFβ1, although T-cell independent B-cell class switching in GC might be mediated by interaction with dendritic cells (DC) and stromal cells, including follicular DC [35].

Also at extrafollicular mucosal sites antibodies can develop both with and without the help of T-cells, the latter involving BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand) [36,37]. Although hypermutation, necessary for affinity maturation, was reported to be minimal at these sites [38], antigen-specific antibody producing B-cells that developed at this site were shown to play a role in prevention of reinfection [39], and to lead to the generation of an IgG and IgA producing B_{Mem} subset [40]. Recombinatorial, transcriptional and signalling events underlying IgA class switching were recently reviewed [32]. Below, we will highlight the immunological parameters that could be a target for the induction of IgA production upon influenza vaccination (Fig. 2).

3.1. Mucosal tissue

The inductive sites of the mucosal immune system include mucosa-associated lymphoid tissue (MALT) as well as local and regional draining lymph nodes. The mechanisms leading to IgA CSR have mostly been studied in the gut and were found to be influenced by the specific environmental factors at this site, mainly created by commensal bacteria and their products [41]. Much less is known about the respiratory tract, which is not populated with as many commensals as the intestinal tract. However, recently it was shown that commensals do play an important role in protection against influenza infection, since antibiotic treatment of mice reduced CD4 T-cell, CD8 T-cell as well as antibody responses, including IgA. Immunity to influenza viruses could be restored by nasal administration of LPS but also by rectal administration of TLR agonists (LPS, CpG, polyI:C) [42]. These findings indicate that signals from distal mucosal regions can support immune priming in the respiratory tract, although it needs further investigation to find whether support from distal regions is preferential over an intact commensal system in the respiratory tract.

3.2. Innate sensing

Influenza viruses interact with several types of pattern recognition receptors, including TLR family members, but also with members of the RIG-I like receptor (RLR) family and the Nod like receptor (NLR) family [43] as well as several members of the C-type lectin receptor family [44]. For vaccination purposes, also members of these receptor families that are not activated by a natural infection have been targeted. For example, flagellin, that activates TLR5, was also shown to promote IgA production and heterosubtypic protection when incorporated in the membrane of influenza VLPs [45]. Similarly, PolyI:PolyC12U, activating TLR3, was shown to induce heterosubtypic protection through IgA antibodies when administered intranasally [23]. Moreover, the effect of this TLR3 ligand was shown to act in a synergistic manner with the TLR-2 ligand zymosan [46].

Recently, several possible models were developed that implicate an essential role for TLR signalling in CSR. Classically, only two signals were described to be necessary to induce CSR in naïve B-cells: presentation of the antigenic peptides on MHC class II molecules after antigen binding to the B-cell receptor and secondly, activation of these B-cells via cytokines and CD40-CD40L interaction with antigen specific T-cells. Currently, TLR signalling

is thought to sometimes provide an important third signal [47] and it has been reported that MyD88 was necessary to induce protection in primary, but not secondary, influenza infection. IgA levels in MyD88$^{-/-}$TRIF$^{-/-}$ mice were reduced in saliva, however, in serum, BALF and nasal wash, levels were similar to those in WT mice and thus induced in a TLR independent manner [48]. In contrast, in another study it was shown that TLRs can play a role in both T-cell dependent and independent IgA responses at both mucosal and systemic levels [49]. This inconsistency might partially be explained by the finding that MyD88 is not only involved in TLR signalling, but it also interacts with TACI (Transmembrane Activator and CAML-Interactor) which is involved in both T-cell dependent as well as independent class switching. Thus, in mice lacking MyD88, both TLR, and TACI signalling will be affected [50].

3.3. APC bridging innate and adaptive immunity

Some antigen presenting cells have been associated with induction of IgA responses, including pDC (plasmacytoid DC), Tip-DC (TNF and Inducible nitric oxygen species (iNOS) Producing DC) and LAPC (late-activator APC).

pDC, highly appreciated in anti-influenza responses for the induction of type 1 interferon, Th1 and cytotoxic responses, were found to also enhance B-cell expansion and differentiation into CD27^{high} plasmablasts upon TLR7 stimulation [51]. Interestingly, pDC were found to be necessary for optimal mucosal IgA and serum IgG production in primary, but not booster influenza vaccination schedules, upon vaccination with live attenuated virus, inactivated whole virus or split virus. In contrast, pDC were not essential for raising a response to live virus [52].

Upon influenza infection, Tip-DC (TNF and inducible nitric oxygen species (iNOS) producing DC) were first known for their production of large amounts of both TNF and NO upon infection with highly virulent strains, thereby inducing tissue damage [53]. However it was recently found that NO – when present in controlled amounts – can induce TGF-βRII expression on B-cells, thereby enabling T-cell dependent IgA class switching. Also, MyD88 signalling downstream of TLR2, 4 and/or 9, needed to induce iNOS, was involved in T-cell independent IgA secretion, in a BAFF and APRIL-dependent manner [54].

Another type of APC that might play an important role was recently identified and designated LAPC, or late-activator APC. Whereas influenza-activated DC are most active around day 3 after infection and induce a response with many Th1 type characteristics, LAPC peak around day 8 after infection and induce a Th2-type polarization, resulting in IgA, IgG1 and IgG2 antibody production, and downregulation of anti-viral Th1-type responses [55].

3.4. Adaptive CD4 T-cell responses

CD4 T-cells are well known for supporting humoral and cellular responses and in addition they can activate innate immunity [28] and display cytolytic potential [56]. The role of CD4 helper T-cells in enhancing B- and CD8 T-cell, immune responses is dependent on the ability of the CD4 T-cells to present antigen on their surface in the context of MHCII molecules as well as the cytokine environment they create.

Importantly, vaccination with peptide variants of the desired epitopes that possess high affinity interactions with the MHC molecules in the host, will increase the amount of these antigens presented on CD4$^+$ T-cells, thereby promoting priming of T and B-cells that interact most efficiently with each other [57]. This could be used to expand the CD4 T-cell repertoire specific for the most genetically conserved regions of influenza HA and NA antigens, thereby enhancing cross-protective neutralizing antibody responses [57]. It was suggested that via priming in the upper respiratory tract, this way also the antigen specificity of IgA antibodies might be influenced by vaccination.

In addition to the MCH molecules, also the cytokine environment created by different T-cell subsets will influence immune responses. The predominant subset of CD4$^+$ T-cells responsible for the generation of high-affinity, class-switched antibodies are follicular helper T-cells (Tfh), that were defined in 2000 by Schaerli et al. as well as Breitfeld et al., based on their surface CXCR5 expression and their key role in antibody production in GC. Tfh are involved in the formation of GC and in the induction of CD40L, IL-2, IL-4, IL-10, TGFβ and IL-21, thereby promoting B-cell proliferation, CSR and somatic hypermutation, resulting in highly specific class switched plasma cells and long-lived memory cells [58,59]. TGFβ was also found to play an important role in IgA class-switching, since mice deficient in TGFβII receptor on B-cells, were hardly producing IgA [60] and S-IgA could not be detected after mucosal vaccination in these mice [61]. Moreover, in the GC, TGFβ and IL-21, produced by Tfh, were found to synergize to stimulate the generation of high numbers of IgA plasmablasts [58].

Recently, it was found that blood circulating cells expressing CXCR5 might be related to Tfh. Three subsets were found; Th1, Th2 and Th17, of which the latter most strongly induced IgA responses [62]. In addition, CXCR5 was also found on a subset of peripheral blood central memory cells and these were proposed to enable quick and efficient secondary antibody mediated immune responses [63].

4. Humoral memory

The essence of vaccination is priming of the immune system with an antigen to induce a quick and effective immune response upon a subsequent encounter of the pathogen bearing that antigen. Success for all current vaccines is based on long-lived antibody production with high affinity, with antibodies shown to be maintained for 75 years after smallpox vaccination [64]. Whether this can be achieved by influenza vaccination needs to be investigated, however, in the serum of people who were naturally infected during the 1918 influenza pandemic, B_{Mem} could be isolated from serum at least up to 70 years after the last encounter [65]. Moreover, these antibodies were also active against the mild A(H1N1)pdm09 [66]. Humoral immune memory is provided by recirculating B_{Mem} and long lived plasma cells, typically residing in the bone marrow [67]. However, much needs to be discovered about the role of B_{Mem} in protection to subsequent influenza infections, regarding for example isotype expression and localization.

In mice, proliferating T-cells were shown to obtain gut- and skin-homing properties during antigen priming in mesenteric and the peripheral lymph nodes, respectively [3]. The factors inducing this are largely unknown, although retinoic acid produced by DC was found to contribute to gut-homing, whereas vitamin D3 metabolites contribute to skin-homing.

It has been stated that probably similar mediators can induce homing to the respiratory tract, with Waldeyer's ring and/or cervical lymph nodes functioning as the antigen stimulation site for T and B-cells with respiratory tract homing properties [3]. Thus, antigens reaching lymph nodes in mucosa-associated lymphoid tissue (MALT) might stimulate mucosal immunity in the same region. Recently it was also found that TGFβ and IL-21, produced by Tfh, not only synergize to induce IgA class switching, but also to simultaneously downregulate CXCR5 and upregulate CCR10 on plasmablasts, enabling their exit from GC and migration towards local mucosa, verifying that Tfh cells play an important role in establishing high-affinity and long-term responses [58]. This was confirmed by a

study that found Tfh to be an important reservoir of memory cells in secondary responses to antigen [68].

B_{Mem} that are produced in GC in the acute phase of infection are known to circulate and to spread to secondary lymphoid tissues. In mice, 8–12 weeks after infection many influenza specific IgA and IgG B_{Mem} were present in these lymphoid tissues, but also in the lung. Upon vaccination, lymph node and lung analysis revealed a higher frequency of IgA B_{Mem} after intranasal compared to intramuscular vaccination, however even upon intranasal vaccination levels of B_{Mem} detected in the lung were very low compared to levels induced by infection [69].

When an intramuscular inactivated vaccine was compared to an intranasal, live attenuated vaccine in a human study, both memory IgG and IgA responses in the circulation were higher after intramuscular administration of the inactivated vaccine, but local memory responses were not determined in this study [70].

Further insight into which homing receptors are involved in B_{Mem} dispersion and how this relates to for example the pathogen, the type of immune activation and the site of induction would be very useful for rational vaccine design. In addition, different types of B_{Mem} might be programmed for specific functions at a specific location. It was recently found that at least two distinct types of IgA B_{Mem} exist: $CD27^+$ and $CD27^-$ IgA B_{Mem}. The former are produced in GC whereas the latter are GC independent and most likely produced locally. The $CD27^-$ B-cells were shown to be induced independent of T-cells, since similar levels of these cells were found in CD40L deficient patients as compared to healthy controls [40]. However, whether these differences are also reflected in their localization and functions, remains to be investigated.

Currently, long-term memory responses induced by influenza vaccines are not tested before use. However, recent studies in mice, ferrets and humans showed that the heterologous protective capacities of prior vaccination depends greatly on the type of vaccine used as well as the type of subsequent vaccination or infection and can be beneficial [71], but also detrimental [72–74]. The latter could have severe consequences in case of a pandemic and was therefore intensively studied using data based on A(H1N1)pdm09 infections. However, conflicting data have been reported and prior vaccination with a seasonal trivalent vaccine has been associated with either increased illness due to A(H1N1)pdm09 [75,76], no association [77–79] or an association with protection of A(H1N1)pdm09 related illness [80–83]. Importantly, all these studies represent association studies and many confounding factors might be unknown, as is stated by the authors as well. Thus no thorough data are available, but improved epidemical studies will pose a great challenge since it is extremely difficult to take the full history of exposure to influenza antigens in humans into account.

Using a different approach, research on human monoclonal antibodies derived from plasma cells or B_{Mem} suggested that vaccination for seasonal influenza would mainly lead to activation of B_{Mem} responsive against dominant epitopes. In contrast, exposure to an antigen representing a major antigenic shift would increase chances of activating and expanding rare heterosubtypic B_{Mem} recognizing highly conserved epitopes, due to absence of competition by pre-existing B_{Mem} recognizing the dominant epitopes [84,85]. Thus, subsequent vaccination with several highly diverse influenza variants might induce good cross-protection. Importantly, the induction of long-term responses should also be studied, since it was shown that only antibodies derived from B_{Mem}, but not from long living plasma cells, were able to strongly neutralize escape mutants from West Nile virus [86].

5. Implications for vaccine design

Seasonal influenza vaccines are currently prepared, based on the prediction of the strain that might cause the epidemic in the following season. These vaccines are mostly injected intramuscularly or subcutaneously and are designed to prevent the onset of the disease induced by the specific vaccine strains, but these vaccines neither induce cross-protection nor prevent infection, since they mostly induce neutralizing IgG antibody in the serum (Fig. 2).

In contrast, influenza vaccines that are currently being designed are mostly based on other mechanisms than induction of IgG, that induce a broader cross-protection, with the ultimate ambition a so called 'universal influenza vaccine'. Several characteristics of the influenza virus as well as the people that should receive the vaccination have to be taken into account. The diverse and more cross-responsive response upon infection compared to current vaccination would suggest that the induction of several, if not all, immunological effectors that can add to cross-protection would more likely be capable to optimally kill viruses with diverse characteristics and at different locations in the body (Fig. 2). Reasoning based on the vaccine recipients would lead to the same conclusion, since people of different ages, sexes as well as genetic and environmental backgrounds will respond differently to vaccination and might rely on different immune mediators for their protection [28,87]. This is in agreement with prospective findings on the key immunological responses induced by the successful yellow fever vaccine, which was identified as 'broad, polyfunctional and persistent, integrating all effector cells of the immune system' [88]. Importantly, different effector cells will not only work next to each other, but will also interact. For example, CD4 T-cells facilitate B-cell responses but B-cells were reported to in their turn also influence CD4 T-cells in several ways [89]. Also IgA was shown to influence T-cells, since $IgA^{-/-}$ mice showed reduced T-cell priming and memory responses upon influenza vaccination, due to impaired APC function that could be overcome with IL-12 [90] (Fig. 2). More knowledge on these interactions will further take vaccine design out of its traditional methodology based on trial-and-error, towards a more rational approach.

Importantly in this respect, natural influenza virus infection was shown to be superior to vaccination with inactivated virus in inducing cross-protection against infection by mutated viruses within a particular subtype of the A-type virus in humans [91–93]. Inactivated virus in its turn has been shown to be more immunogenic than split vaccines, which is in agreement with the general finding that effectiveness and safety of vaccines are usually inversely correlated.

Thus, both whole virus particles as well as split-product seasonal vaccines can induce strong protection against the homologous virus [94]. However, heterosubtypic immunity is not observed when vaccination is performed using an ether-split vaccine, whereas in the same study administration of an inactivated whole virion vaccine induced a broad spectrum of heterosubtypic immunity [25]. The stronger immunogenicity of the inactivated whole virion vaccine in mice was likely due to the stimulation of innate immunity by genomic single stranded RNA, via TLR7 [95,96]. Since most viruses produce dsRNA during replication [97], synthetic dsRNA can likely act as a partial molecular mimic of viral infection.

This has been confirmed in a study where intranasal administration of an ether-split vaccine from PR8 (a H1N1 type influenza strain) and poly(I:C) adjuvant induced a strong anti-HA IgA and IgG response in nasal washes and serum, respectively, while vaccination without poly(I:C) induced very little response. In addition, administration of either an A/Beijing (H1N1) or A/Yamagata (H1N1) vaccine which are antigenically different from A/PR8, in the presence of poly(I:C) conferred complete protection against A/PR8 virus challenge in a mouse model of nasal infection, suggesting that intranasal vaccination with poly(I:C) adjuvant confers cross-protection against variant viruses [15]. Clinically safe when administered intravenously, with intranasal administration currently in the pre-clinical phase and recently shown to be a potent

inducer of innate immune responses upon subcutaneous administration [98], dsRNA, poly(I:C_{12}U)(Ampligen), was investigated as a dsRNA adjuvant for intranasal avian influenza vaccines [22].

The stronger immunogenicity of the live virus compared to the whole inactivated vaccine may be caused by many mechanisms other than stimulation of TLR7 or 3, such as additional receptors involved or a different biodistribution or kinetic profile of live virus compared to inactivated vaccines. The former might be mimicked by using a ligand for those receptors as an adjuvant, the latter two might possibly be mimicked by the use of different carriers for the antigens that will influence kinetics as well as biodistribution [99].

While we are currently still learning from influenza virus infections, ultimately we would like to design vaccines that outclass natural infections. This might be achieved by careful selection of highly conservative parts of influenza membrane proteins, in combination with several adjuvants that together will activate the required broad spectrum of tissues and cells.

A very promising combination might be nanoparticles, mostly associated with enhanced CD8+ T-cell responses, and TLR ligands, that together can induce very strong and broad humoral responses via induction of GC formation and expansion of Tfh cells [100]. For the rational design of effective vaccines directed against different pathogens, increased understanding of the mechanisms of single as well as combinations of adjuvants in great detail [98,100] will be indispensable.

Notably, recent clinical trials revealed that the intranasal administration of a whole inactivated influenza virus to healthy human subjects, without adjuvant but with a prime-boost regimen, induced high levels of nasal neutralizing antibodies that consisted primarily of polymeric IgA (unpublished data). Whether the absence of adjuvant was less important in human subjects because of the pre-existence of (cross-)protective memory due to a history of infections and/or vaccinations, we are currently investigating.

In conclusion, the induction of IgA will broaden the immune response induced by vaccines, by introducing local immune responses, adding to cross-protection, balancing pro-inflammatory responses and making memory similarly more diverse (Fig. 2). That IgA alone will most probably not be able to induce full protection in case of a heterosubtypic infection could actually be an advantage, since partial protection by IgA will reduce the viral load, while leaving enough space for the cellular immune system to get primed. This way, innate, humoral and cellular responses will all be activated, resulting in the strongest renewal of the immunological memory and ensuring the best possible preparedness for the next influenza virus that will be encountered.

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Characterization of Neutralizing Antibodies in Adults After Intranasal Vaccination With an Inactivated Influenza Vaccine

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The levels and properties of neutralizing antibodies in nasal wash and serum collected from five healthy adults were examined after intranasal administration of an A/Uruguay/716/2007 (H3N2) split vaccine (45 μg hemagglutinin (HA) per dose; five doses, with an interval of 3 weeks between each dose). Prior to the assays, nasal wash samples were concentrated so that the total amount of antibodies was equivalent to about 1/10 of that found in the natural nasal mucus. Vaccination induced virus-specific neutralizing antibody responses, which increased with the number of vaccine doses given. Neutralizing antibodies were produced more efficiently in the nasal passages than in the serum: A ≥4-fold increase in nasal neutralization titres was observed after the second vaccination in four out of five subjects, whereas a rise in serum neutralization titres was observed only after the fifth vaccination. Nasal and serum neutralizing antibodies were mainly found in the polymeric IgA and monomeric IgG fractions, respectively, after gel filtration. Taken together, these results suggest that intranasal administration of an inactivated split vaccine induces high levels of nasal neutralizing antibodies (primarily polymeric IgA) and low levels of serum neutralizing antibodies (primarily monomeric IgG). ***J. Med. Virol. 84:336–344, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: influenza; vaccine; neutralizing antibody

INTRODUCTION

To prevent influenza, protective immunity must be induced in advance by administration of a vaccine. Currently available inactivated vaccines, detergent disrupted split-viruses, or purified glycoproteins (surface antigen vaccines) are given via parenteral injection [Murphy and Webster, 1996]. Parenteral vaccination, that is, vaccination via the non-mucosal route, induces serum IgG antibodies, which are highly protective against homologous virus infection, but less effective against heterologous virus infection. Thus, intramuscular vaccination of seasonal influenza vaccine would be less effective in protecting against a heterologous virus epidemic.

A large number of studies show that the protective immunity induced by influenza virus infection is mainly mediated by secretory IgA (S-IgA) and IgG antibodies within the respiratory tract. S-IgA is carried to the mucus by transepithelial transport, while serum IgG is transported from the serum to the mucus by diffusion [Murphy and Clements, 1989; Brandtzag et al., 1994; Murphy, 1994; Asahi et al., 2002; Asahi-Ozaki et al., 2004]. S-IgA in the upper respiratory tract prevents viral infection, while IgG supports S-IgA-mediated protection by neutralizing newly-generated viruses [Ito et al., 2003; Renegar et al., 2004]. IgG is the main antibody involved in anti-viral protection in the lungs [Ramphal et al., 1979; Palladino et al., 1995; Renegar et al., 1998; Ito et al., 2003]. Also, polymeric S-IgA neutralizes viruses more effectively than monomeric IgA or IgG [Taylor

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and Dimmock, 1985; Renegar et al., 1998]. The polymeric nature of S-IgA also explains why S-IgA cross-reacts with variant influenza viruses to a greater extent than serum IgG [Tamura et al., 1990, 1991, 1992; Asahi-Ozaki et al., 2004]. Thus, intranasal administration of an inactivated influenza vaccine is advocated to elicit S-IgA and IgG responses and improve the protective efficacy of current vaccination procedures [Tamura and Kurata, 2004; Tamura et al., 2005, 2010].

Several clinical trials have examined the induction of both S-IgA and IgG following intranasal administration of inactivated influenza vaccines, either with or without adjuvant [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The antibody responses after intranasal administration of inactivated influenza vaccines were assessed by measuring hemagglutination inhibition (HI) titres in the serum, and anti-hemagglutinin (HA) IgA and IgG titres in nasal wash samples. They did not measure the titre of neutralizing antibodies, which is considered to be a better criterion for functional protective antibodies. Neutralization titres can directly inhibit the complex process involved in virus replication, which include virus attachment and entry to the host cells, and release of newly-synthesized virus from the infected cells in tissue culture. In addition, a previous study found that HI titres were lower, or higher, than the corresponding neutralization titres, depending on a strain of influenza A or B virus used for the assay [Okuno et al., 1990], whereas other studies show that anti-H5 HI antibodies fail to detect H5N1 viruses [Lu et al., 1982; Rowe et al., 1999]. Thus, neutralizing antibody responses following intranasal administration of an inactivated influenza vaccine remain to be fully characterized.

Therefore, the aim of the present study was to examine the levels and properties of neutralizing-antibodies in nasal wash and serum samples from healthy adults after intranasal administration of an inactivated vaccine (five doses, with an interval of 3 weeks between each dose). The inactivated vaccine used in this study was a concentrated split-virus vaccine (containing 45 μg HA per dose), prepared from the A/Uruguay/716/2007 (H3N2) strain. A concentrated split-virus vaccine was chosen because the vaccine has already been shown to induce mucosal antibody responses after intranasal vaccination [Kuno-Sakai et al., 1994]. To ensure that neutralization titres specific for the A/Uruguay/716/2007 virus were assayed at equivalent levels in both serum and nasal wash samples, the neutralization titres were measured using concentrated nasal wash samples (1 mg/ml total protein) that contained approximately 1/10 of the IgA found in undiluted mucus [Kurono and Mogi, 1987]. The properties of the neutralizing IgA and IgG antibodies induced by intranasal vaccination were then examined, and their relative levels and molecular size were determined.

MATERIALS AND METHODS

Subjects

Five healthy male subjects (P1, P2, P3, P4, and P5) were enrolled in the study (aged 22, 32, 42, 42, and 68 years, respectively, at the time of the first vaccination). All participants had already acquired some degree of immunity to H1N1 and H3N2 influenza A virus subtypes after previous exposure to these viruses and/or as a result of previous vaccinations. Each subject provided informed consent and the study protocol and other relevant documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Virus and Vaccine

The A/Uruguay/716/2007 (A/Uruguay; H3N2) influenza virus strain was propagated in the allantoic cavity of 10-day-old embryonated hen's eggs and purified from the allantoic fluid. The $TCID_{50}$ (50% infectious dose in tissue culture) of the virus was estimated as described previously [Tobita et al., 1975; Kadowaki et al., 2000]. In brief, 10-fold serial dilutions of the allantoic fluid containing the virus were inoculated into Madin-Darby canine kidney (MDCK) cells (ATCC No. CCL-34) cells in 96-well culture plates and incubated for 4 days at 37°C in a 5% CO_2 humidified atmosphere. The cytopathic effects in the virus-containing wells were monitored under a microscope and the $TCID_{50}$ was calculated using the Reed–Muench method. The split product virus vaccine was supplied by the Research Foundation for Microbial Disease of Osaka University (BIKEN, Kanonji, Japan). The vaccine was prepared from purified viruses, which were sedimented through a linear sucrose gradient according to the manufacturer's protocol. The viruses were then treated with ether and formalin according to the manufacturer's protocol, which was based on the method of Davenport et al. [1964]. The concentrated split vaccine containing 45 μg HA was the product of a process used to prepare a trivalent vaccine comprising A/H1N1, A/H3N2, and B type vaccines, each containing 15 μg HA.

Vaccinations

All participants were immunized intranasally with a threefold concentrated split H3N2 virus vaccine (A/Uruguay, containing 45 μg HA). Each received five doses, with an interval of 3 weeks between each dose. Intranasal vaccination was performed by spraying 0.25 ml of the split vaccine into each nostril (0.5 ml total) using an atomizer (Keytron, Ichikawa, Japan). The mean droplet diameter was 56.5 μm, ranging in size between 10 μm and 90 μm.

Nasal Wash and Serum Samples

About 100 ml of nasal wash was collected from each participant in polypropylene tubes by washing the