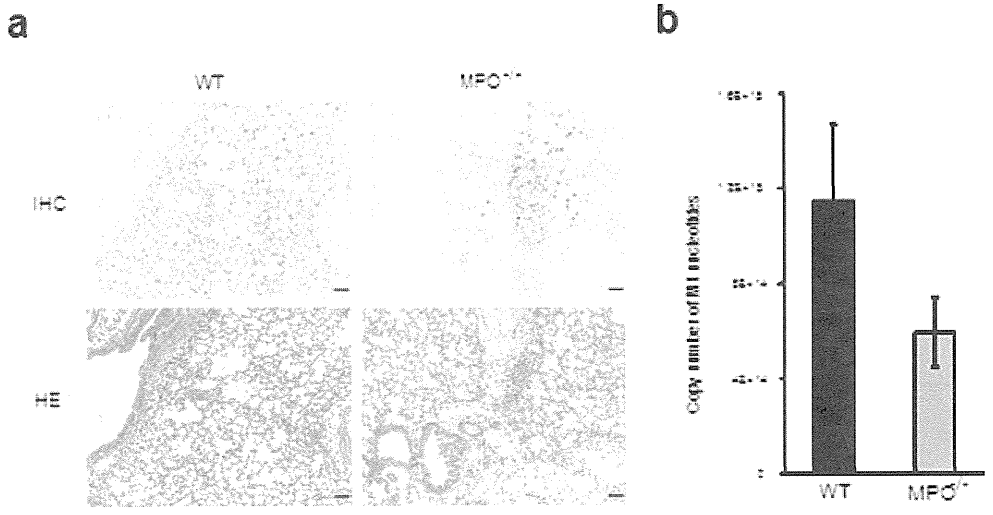

Fig. 6. Viral load in MPO$^{-/-}$ mice. (a) Immunohistological detection of InfA-NP in the lung from wild-type and MPO$^{-/-}$ mice infected with PR-8 at 1400 p.f.u. More viral antigens were found predominantly in bronchiolar and alveolar epithelial cells in the lung tissue of wild-type than in that of MPO$^{-/-}$ mice on 3 dpi. Specimens stained with HE are shown as serial sections. Data shown are representative of results from more than two individuals. Scale bars, 500 μm. (b) Copy number of matrix 1 (M1) nucleotides of PR-8 virus in lung tissue from wild-type and MPO$^{-/-}$ mice infected at 1400 p.f.u. on 3 dpi. Data are expressed as the mean ± SD of results from two individuals.

contribute to the clearance of influenza virus by phagocytosing virus-infected apoptotic cells (10). The blockade of MCP-1 not only significantly increases viral titer, but also enhances alveolar epithelial disruption due to lack of macrophage-mediated function (27,38,39). This indicates that macrophages increased by influenza virus infection are an important factor to reduce viral load and lung damage. It is thought that macrophages chemoattracted by MCP-1 are not associated with aggravation of lung damage during influenza virus infection.

The excessive generation of HOCl mediated by MPO causes oxidative tissue damage (10,12,14,16). The inhibition of neutrophil influx attenuates lung inflammation and damage with suppression of MPO activity in the BALF during influenza virus infection (14). In our study, inflammatory damage in the lung was reduced by the absence of MPO activity with suppression of total protein leakage in the BAL. Interestingly, the absence of MPO rescued the expression levels of claudins 9 and 18–1 in the lung, independently of claudins 2 and 4. These observations suggest that claudins 9 and 18–1 are affected by HOCl produced by MPO, and that maintenance of both claudins prevents lung tissue protein leakage during the infection. In addition, increasing MIP-1β and decreasing GM-CSF production were observed by the absence of MPO. MIP-1β recruits macrophages into the lung during pathogenic pneumonia (40,41), and blockade of GM-CSF inhibits recruitment of neutrophils (42–43). In our study, however, there were no significant differences of neutrophil or macrophage cell number in BALF between MPO$^{-/-}$ and wild-type mice on 4 dpi (data not shown). Both cytokines are not likely to be associated with recruitment of leukocyte populations. Inflammatory cytokines influence lung TJ via claudin alteration (7–9). Although precise roles of these cytokines induced by the absence of MPO are not elucidated, these cytokines might regulate claudins 9 and 18–1 to maintain TJ function during the influenza virus infection.

The absence of HOCl by MPO deficiency, however, did not show a survival effect. The concentration of total protein in BALF of MPO$^{-/-}$ mice was still higher than that of uninfected mice. These observations suggest that there are other damage factors, such as elastase which is also derived from activated neutrophils and is implicated in ALI by its proteolytic activity as well as in host defense (44–46). Such potential factors may affect claudins 9 and 18–1. Actually, the deletion of MPO did not completely rescue the expression of these claudins, suggesting the association with other factors, in addition to MPO. In our experiment, we depended on administration of a high concentration of virus for preparation of the ARDS model (1400 p.f.u./mouse). Using this mouse model, remarkable chemokines and several roles of MPO in the lung damage were elucidated. However, we speculate that viral concentration is too great to highlight a survival effect in MPO$^{-/-}$ mice. In fact, when mice were infected at 320 p.f.u./mouse, the MPO$^{-/-}$ mice showed a trend of survival prolongation in comparison with wild-type mice (data not shown).

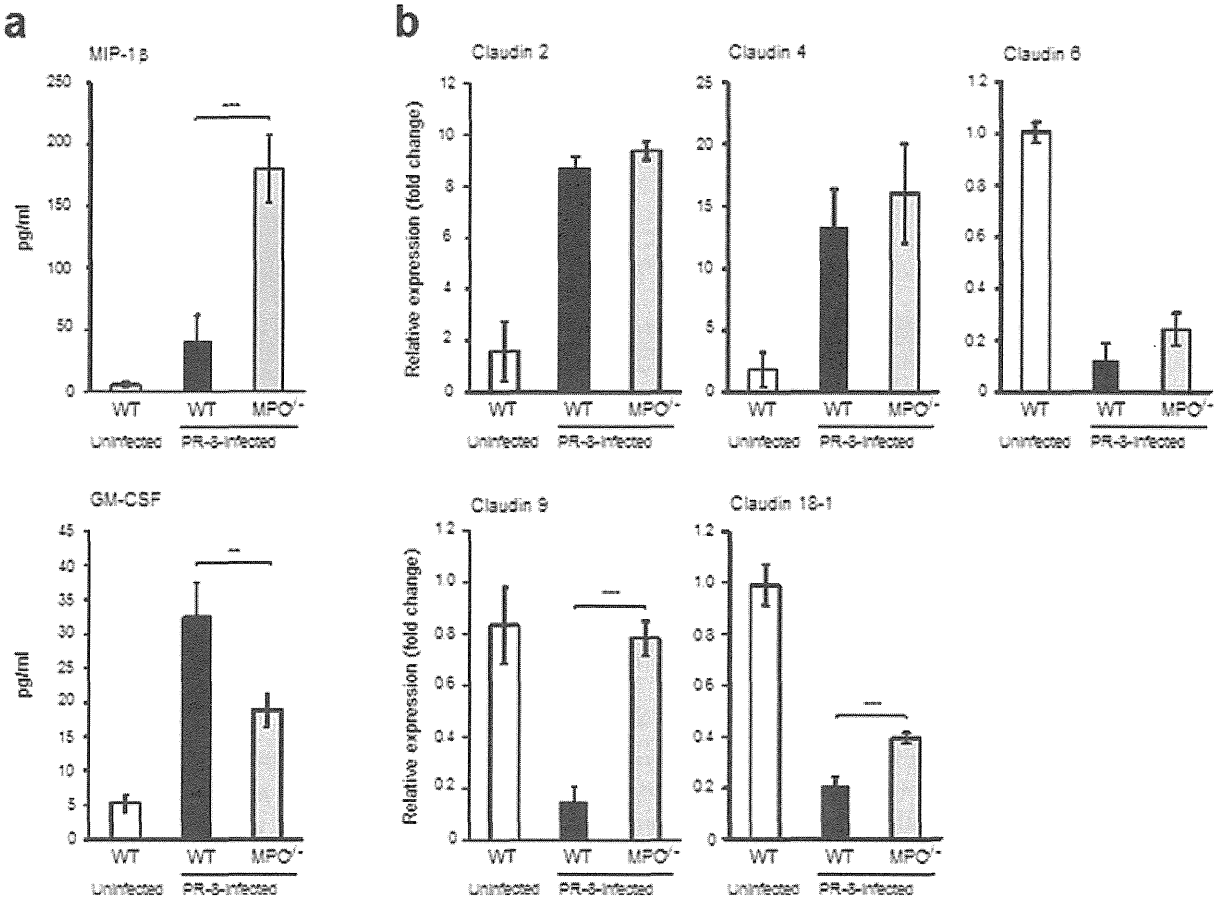

Fig. 7. Differential production of cytokines in MPO$^{-/-}$ and wild-type mice. (a) Cytokine protein in BALF of each mouse strain on 4 dpi. Mice were infected with PR-8 at 1400 p.f.u. (b) Gene expression of claudins in the lung tissue infected with PR-8 at 1400 p.f.u. The expression level of each claudin gene was compared between MPO$^{-/-}$ and wild-type mice on 4 dpi. Data are expressed as the mean ± SD of results from three individuals. $^{*}P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test).

Low concentration of virus for the infection may result in the survival difference between the two mouse strains.

MPO is involved in virus clearance of influenza, mediating generation of reactive oxygen intermediates (10,13). However, in the present study, the absence of MPO reduced the viral load in the lung. It is difficult to explain these apparently conflicting phenomena. We speculate that histological disruption of alveoli in wild-type mice is compatible with viral spread with respiratory air in comparison with conditions of MPO$^{-/-}$ mice, as MPO-mediated HOCl is associated with damage via claudin alteration. Inhibition of lung damage by the absence of MPO may prevent viral spread by the maintenance of anatomical structures and barrier function in MPO-deficient mice.

In summary, we have established an animal model of fulminant ARDS induced by influenza virus infection. In this model, KC, MIP-2, and RANTES play pivotal roles in recruitment of leukocytes leading to pneumonia at the early phase of influenza virus infection. Neutrophil MPO mediating HOCl generation potentially plays a role in inflammatory damage of the lung by alteration of limited claudins, as well as by influencing viral expansion during the infection. We propose that the cause of fulminant ARDS induced by the influenza virus is the expression of acute inflammatory mediators and damage factors including MPO, which induces oxidative stress resulting in viral spread.

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DISCLOSURE

The authors have no financial conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Fig. S1. Production of cytokines in plasma in uninfected and infected mice. Cytokine and chemokine proteins were quantitated by Multi-plex analysis. Productions of IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-17, GM-CSF, and MIP-1α were at a notably low level (data not shown). Data in the figure are expressed as mean ± SD of results from three individuals. $^*P < 0.05$ and $^{**}P < 0.01$ (Student's *t*-test).

Table S1. Primer set for quantitative real-time PCR analysis.

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ORIGINAL ARTICLE

Efficacy of inhaled N-acetylcysteine monotherapy in patients with early stage idiopathic pulmonary fibrosis

SAKAE HOMMA,[1] ARATA AZUMA,[2] HIROYUKI TANIGUCHI,[6] TAKASHI OGURA,[7] YOSHIRO MOCHIDUKI,[8] YUKIHIKO SUGIYAMA,[9] KOICHIRO NAKATA,[3] KUNIHIKO YOSHIMURA,[4] MASAHIRO TAKEUCHI,[5] SHOJI KUDOH[2] AND THE JAPAN NAC CLINICAL STUDY GROUP

[1]*Department of Respiratory Medicine, Toho University Omori Medical Center,* [2]*Fourth Department of Internal Medicine, Nippon Medical School,* [3]*Nakata Clinic,* [4]*Department of Respiratory Medicine, Toranomon Hospital* and [5]*Department of Biostatistics, Kitasato University, Tokyo,* [6]*Department of Respiratory and Allergy, Tosei General Hospital, Aichi,* [7]*Department of Respiratory Medicine, Kanagawa Cardiovascular and Respiratory Center, Kanagawa,* [8]*Department of Internal Medicine, NHO Himeji Medical Center, Himeji, and* [9]*Division of Pulmonary Medicine, Jichi Medical University, Tochigi, Japan*

Correspondence: Arata Azuma, Department of Internal Medicine, Nippon Medical School, Tokyo, 113-8602, Japan. Email: a-azuma@nms.ac.jp

The members of the Japan NAC Clinical Study Group are: S. Kudoh and A. Azuma (Fourth Department of Internal Medicine, Nippon Medical School, Tokyo), S. Homma (Department of Respiratory Medicine, Toho University Omori Medical Center, Tokyo), H. Taniguchi (Department of Respiratory and Allergy, Tosei General Hospital, Aichi), T. Ogura (Department of Respiratory Medicine, Kanagawa Cardiovascular and Respiratory Center, Kanagawa), Y. Mochizuki (Department of Internal Medicine, NHO Himeji Medical Center, Himeji), Y. Sugiyama (Division of Pulmonary Medicine, Jichi Medical University, Tochigi), K. Nakata (Nakata Clinic, Tokyo), M. Munakata (Department of Pulmonary Medicine, Fukushima Medical University, Fukushima), T. Nukiwa (Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Miyagi), Y. Ishii (Department of Pulmonary Medicine and Clinical Immunology, Dokkyo Medical University School of Medicine, Tochigi), K. Yoshimura (Department of Respiratory Medicine, Toranomon Hospital, Tokyo), M. Oritsu (Department of Respiratory Medicine, Japanese Red Cross Medical Center, Tokyo), Y. Yoshizawa (Department of Integrated Pulmonology, Tokyo Medical and Dental University, Tokyo), H. Takizawa (Department of Internal Medicine, Teikyo University School of Medicine, Tokyo), K. Ohta (Department of Internal Medicine, Teikyo University School of Medicine, Tokyo), E. Suzuki (Department of Internal Medicine, Niigata University, Niigata), K. Chida (Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu), Y. Inoue (Kinki-Chuo Chest Medical Center, Osaka), N. Kohno (Department of Molecular and Internal Medicine, Hiroshima University, Hiroshima), Y. Nishioka (Department of Internal Medicine and Molecular Therapeutics, Tokushima University, Tokushima), H. Hamada (Department of Integrated Medicine and Informatics, Ehime University Graduate School of Medicine, Ehime), S. Kohno (Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki), M. Suga (Department of Respiratory Medicine, Saiseikai Kumamoto Hospital, Kumamoto), Y. Taguchi and S. Noma (Tenri Hospital, Tenri), H. Takahashi (Third Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo), M. Kanazawa (Department of Respiratory Medicine, Saitama Medical School, Saitama), F. Sakai (Department of Diagnostic Radiology, Saitama International Medical Center, Saitama Medical University, Saitama), K. Tomii (Department of Respiratory Medicine, Kobe City Medical Center General Hospital, Kobe), Y. Tomioka (Department of Respiratory Medicine, Kobe City Medical Center West Hospital, Kobe), and M. Takeuchi (Department of Biostatics, Kitasato University, Tokyo).

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SUMMARY AT A GLANCE

A multicentre, prospective, randomized, controlled trial was conducted to assess the efficacy of monotherapy with inhaled N-acetylcysteine (NAC) in patients with IPF. Exploratory analyses demonstrated that NAC monotherapy stabilized the serial decline in FVC in some patients without the use of immunosuppressive or anti-fibrotic agents.

ABSTRACT

Background and objective: **Idiopathic pulmonary fibrosis (IPF) is a fatal disorder for which there are currently no specific or effective medical treatments. A multicentre, prospective, randomized, controlled clinical trial was conducted to assess the efficacy of inhaled N-acetylcysteine (NAC) monotherapy in Japanese patients with early stage IPF.**

Methods: **Eligible patients had well-defined IPF of mild-to-moderate severity, with no desaturation on exercise. Of 100 patients screened, 76 were randomly assigned to an NAC treatment group (group A; $n = 38$) that received 352.4 mg of NAC by inhalation twice daily or to a control group (group B; $n = 38$) that received no therapy. The primary endpoint was the change from baseline in forced vital capacity (FVC) at 48 weeks.**

Results: **There were no significant overall differences in the change in FVC between groups A and B. Post hoc exploratory analyses showed that NAC therapy was associated with stability of FVC in (i) a subset of patients with initial FVC <95% of predicted ($n = 49$; difference in FVC decline 0.12 L; $P = 0.02$) and (ii) in patients with initial diffusing capacity of carbon monoxide <55% of predicted ($n = 21$; difference in FVC decline 0.17 L; $P = 0.009$).**
Conclusions: **These findings indicate that NAC monotherapy may have some beneficial effect in patients with early stage IPF. Further trials in more select IPF populations with progressive disease are required to prove the efficacy of inhaled NAC.**

Key words: idiopathic pulmonary fibrosis, inhaled N-acetylcysteine, monotherapy, oxidant–antioxidant imbalance, randomized controlled trial.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a devastating, progressive and fatal disorder. It is characterized by the findings on high-resolution computed tomography (HRCT) and histological features of usual interstitial pneumonia in adults over 50 years of age, with exertional dyspnoea and abnormal pulmonary function tests (PFT).[1] An oxidant–antioxidant imbalance may contribute to the disease process in IPF.[2] Evidence that antioxidants are decreased in bronchoalveolar lavage fluid from patients with IPF is supported by the observation of an approximately fourfold decrease in glutathione (GSH) levels in IPF patients compared with healthy control subjects.[3] Therefore, it is logical to consider an antioxidant-based treatment strategy for patients with IPF. It is possible to raise GSH levels in bronchoalveolar lavage fluid of patients with IPF by administration of GSH aerosols, and this in turn is associated with a significant reduction in the release of oxidants by alveolar macrophages.[4]

N-acetylcysteine (NAC) is a tripeptide (γ-glutamyl-cysteinyl-glycine) that is not only a precursor of GSH but also directly scavenges oxygen-free radicals.[5] Furthermore, inhibition of transforming growth factor-β signalling or its direct modification by NAC may be beneficial in IPF, the pathogenesis of which is associated with excessive amounts of this growth factor.[6]

NAC at a high oral dose of 600 mg thrice daily or when administered intravenously has been shown to replenish GSH levels in the epithelial lining fluid, as well as intracellularly, in patients with IPF.[7–9] Based on these and other findings, a randomized controlled trial of NAC, 600 mg thrice daily, administered with prednisone and azathioprine as compared with placebo plus prednisone and azathioprine was performed in 155 patients with IPF and showed significant beneficial effects on the declines in vital capacity (VC) and diffusing capacity of carbon monoxide (DL_{CO}) after 1 year.[10]

However, NAC may not produce a sustained increase in GSH levels sufficient to increase the antioxidant capacity of the lungs even when administered in high oral doses (600 mg thrice daily).[11] Furthermore, NAC itself is not detected in bronchoalveolar lavage fluid when administered orally (200 mg thrice daily).[12] In contrast, when administered as an aerosol, NAC acts directly as an antioxidant in the alveoli in addition to increasing GSH levels.[13] Therefore, inhalation of NAC may attenuate inflammation and lung fibrosis more effectively.

The aim of this study was to assess the efficacy and safety of monotherapy with inhaled NAC in patients with early stage IPF by conducting a prospective, randomized, controlled clinical trial at 27 centres in Japan.

METHODS

Study subjects

Between January 2005 and February 2007, patients with well-defined IPF participated in a prospective, controlled, randomized, multicentre, clinical trial at 27 sites in Japan. The protocol was approved by the Institutional Review Board at each centre, and written informed consent was obtained from all participants prior to enrolment. Ongoing efficacy and safety results were reviewed at 6-month intervals by an independent Data and Safety Monitoring Board.

IPF was diagnosed in accordance with the American Thoracic Society/European Respiratory Society Consensus statement[14] and the guidelines of the fourth version of the Japanese clinical diagnostic criteria for idiopathic interstitial pneumonia.[15] While histological evidence of usual interstitial pneumonia was not mandatory, HRCT evidence of a usual interstitial pneumonia pattern in the appropriate clinical setting was required for all patients. A usual interstitial pneumonia pattern was defined as basal predominant, subpleural reticular abnormality with traction bronchiectasis and honeycomb cysts without atypical features of usual interstitial pneumonia.[1,16,17] The presence of other typical clinical features, including bibasilar inspiratory fine crackles, abnormal PFT[14] and increased serum levels of markers of pneumocyte injury (Krebs von den Lungen-6 (KL-6) and surfactant proteins A and D),[18–22] was also a required criteria.

Eligible patients were aged between 50 and 79 years and had firm clinical and radiological diagnoses of IPF, with the severity of disease classified as stage I or stage II, and a lowest arterial oxygen saturation value of >90% during the 6-min walk distance (6MWD) test. Partial arterial oxygen concentration at rest and desaturation during the 6MWD test have been shown to be prognostic factors in IPF patients.[23,24] In Japan, classification of severity of IPF (stages I–IV) has been used for decisions regarding subsidization of medical care. The stages of severity of IPF are as follows: stage I (partial arterial oxygen concentration ≥ 80 mm Hg at rest), stage II (partial arterial oxygen concentration 70–80 mm Hg at rest), stage III (partial arterial oxygen concentration 60–70 mm Hg at rest) and stage IV

(partial arterial oxygen concentration < 60 mm Hg at rest). For patients with stage II or stage III disease, if arterial oxygen saturation during a 6MWD test is <90%, then severity should be increased by one stage.[15] This classification is well correlated with survival of IPF patients.[25] Therefore, in this study, eligible patients with stage I or stage II disease and no desaturation during the 6MWD test were considered to be in the early stage of IPF.

The exclusion criteria were an improvement in symptoms during the preceding 3 months; use of NAC, immunosuppressive agents, oral prednisone or pirfenidone; and clinical suspicion of idiopathic interstitial pneumonia other than IPF.[1]

Efficacy endpoints

The primary endpoint for the effect of NAC was defined as the absolute change in forced vital capacity (FVC) from baseline to 48 weeks. The secondary endpoints were changes in the lowest arterial oxygen saturation, 6MWD and PFT parameters, including VC, % predicted VC, total lung capacity, % predicted total lung capacity, DL_{CO} and % predicted DL_{CO}. Other secondary endpoints were changes in serum markers of pneumocyte injury, including KL-6, and surfactant proteins D and A; disease progression as determined by HRCT; subjective changes in symptoms such as dyspnoea; and adverse events.

HRCT scans were performed at baseline and at 24-week intervals in accordance with a predetermined protocol. Two expert pulmonary radiologists independently evaluated the patterns of lung fibrosis on HRCT scans. Progression of disease was assessed on the basis of HRCT findings by consensus between the site investigator and one of the radiologists. Worsening of disease was defined as progression in the extent of fibrosis (honeycombing and reticular opacity) and ground-glass opacity as compared with baseline. Stable disease was defined as no change with respect to the extent of fibrosis (honeycombing and reticular opacity) and ground-glass opacity. Improvement was defined as a decrease in the extent of ground-glass opacity as compared with baseline.

Serum KL-6 and surfactant protein D levels were measured to assess the changes in blood levels, and Fletcher, Hugh-Jones classification scores[26] were determined to assess patient dyspnoea during activities of daily living. Patients were categorized as 'improved', 'stable' or 'deteriorated' with respect to these parameters (changes of 20% for KL-6 and surfactant protein D, or one grade for the Fletcher, Hugh-Jones dyspnoea classification score).

Patients were randomly assigned to the NAC or control groups (1:1). Fifty-one patients designated as the NAC-treated group were treated twice daily by inhalation of 352.4 mg of NAC, diluted with saline to a total volume of 4 mL, using microair nebulizers and vibration mesh technology (NE-U22, Omron, Tokyo, Japan), which improves lung deposition, as compared with inefficient jet nebulizer systems.[27,28] Microair nebulizers produce aerosol particles with diameters of 1–8 μm with a 50% particle diameter of 5.0 μm. Forty-nine patients, who were designated as the non-NAC-treated control group, received no treatment (or placebo) for 48 weeks.

Statistical analysis

Changes from baseline values were compared by the Wilcoxon test. Categorical variables were compared using Fisher's exact test. Continuous variables were compared using the Wilcoxon test. Analyses of incidences were performed using Fisher's exact test. For missing values, the principle of last observation carried forward was adopted. Analyses of changes in FVC from baseline were performed by analysis of covariance using the respective baseline measurements as covariates. Analyses of changes in other PFT and serum levels of markers of interstitial pneumonia were also performed by analysis of covariance. Post hoc subgroup analyses were performed for patients with initial FVC values <95% of predicted or initial DL_{CO} values <55% of predicted. *P* values were considered to be statistically significant when <0.05.

RESULTS

Characteristics of the patients

One hundred patients were randomized to either the NAC or control groups between January 2005 and February 2007. Of these 100 patients, 90 were included in the full data set for assessment of safety. Seventy six of these 90 patients were included in the data set for assessment of efficacy. Reasons for exclusion from the study among the 100 patients were non-administration of NAC in six patients and missing data for one patient in the NAC group, and protocol violation in one patient and missing data for two patients in the control group. Reasons for exclusion from the study among all patients in the full analysis set are listed in Table 1. There were no significant differences between the two groups with respect to the number of subjects excluded from participation.

Table 1 Reasons for exclusion of patients from the full analysis set

Reasons for exclusion	Number of patients (%)	
	NAC ($n = 44$)	Control ($n = 46$)
Total number	6 (13.6)	8 (17.4)
Acute exacerbation	1 (2.2)	4 (8.7)
Progression of disease	0 (0)	1 (2.2)
Pneumonia	1 (2.3)	0 (0.0)
Death	0 (0)	0 (0)
Patient's request for discontinuation protocol violation and others	4 (9.1)	3 (6.5)

NAC, N-acetylcysteine.

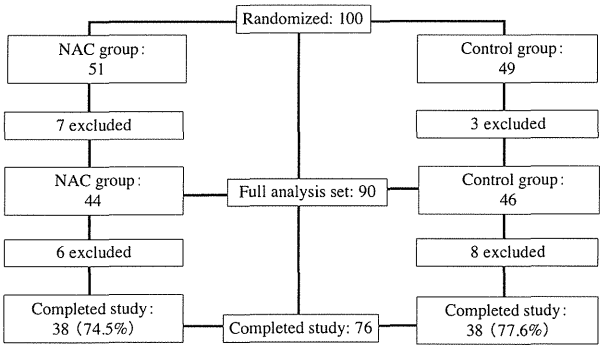


Figure 1 Diagram showing randomization and selection of patients with idiopathic pulmonary fibrosis who were treated or not treated with N-acetylcysteine (NAC).

During the 48 weeks of the study, acute exacerbations of IPF occurred in 8.7% of patients in the control group (4/46) and in 2.2% of patients in the NAC group (1/44). In total, data for 24 patients was excluded from the analysis, and 38 patients in each group completed the study (Fig. 1).

The demographical characteristics and baseline parameters for both groups of patients who completed the study were similar (Table 2), and there were no significant differences between the groups. None of the patients had received any prior treatment.

Overall effects for primary endpoint

The mean changes in FVC, based on analysis of covariance, were decreases of 90 mL and 150 mL at 48 weeks in the NAC and control groups, respectively, with the difference of 63 mL being nonsignificant (Fig. 2). Analysis of the incidence of serial decline in FVC at 48 weeks showed that the percentage of patients with a >10% decline in FVC was 36.4% lower in the NAC group; the percentage of patients with a <10% decline in FVC was 14.8% greater in the NAC group than in the control group ($P = 0.42$).

Overall effects for secondary endpoints

Changes in the lowest arterial oxygen saturation; 6MWD; PFT parameters including VC, % predicted VC, total lung capacity, % predicted total lung capacity, DL_{CO}, and % predicted DI_{CO}; and serum markers of pneumocyte injury, including KL-6, and surfactant protein D and A, showed no significant differences between the NAC and control groups.

The proportion of patients who had improved or were stable at 48 weeks, as assessed by HRCT imaging, was 88.6% for the NAC group and 78.1% for the control group ($P = 0.33$) (Table 3). There were no differences in the extent or severity of the honeycomb pattern on HRCT.

There was improvement or stability of dyspnoea at 48 weeks in 86.8% of patients in the NAC group and 84.2% of patients in the control group ($P = 1.00$) (Table 4).

Post hoc exploratory analyses

Exploratory analysis of primary efficacy showed that in a subset of patients with initial FVC <95 % of predicted, the mean changes in FVC were decreases of 40 mL and 160 mL in the NAC and control groups, respectively (baseline % predicted VC: 79.6 ± 11.1% vs 82.0 ± 10.8%), with the difference being significant ($P = 0.021$) (Fig. 3a). In a subset of patients with initial DL_{CO} <55% of predicted, the mean changes in FVC were decreases of 90 mL and 260 mL in the NAC and control groups, respectively (baseline % predicted DL_{CO}: 41.7 ± 10.2% vs 42.6 ± 7.1%), with the difference being significant ($P = 0.009$) (Fig. 3b). Furthermore, among a subset of patients with initial FVC <95 % of predicted, the percentage of patients with a >10% decline in FVC was 47.9% lower, whereas the percentage of patients with a <10% decline in FVC was 22.5% higher in the NAC group compared with the control group ($P = 0.32$). Among a subset of patients with initial DL_{CO} <55% of predicted, the percentage of patients with a >10% decline in FVC was 76.8% lower, whereas the percentage of patients with a <10% decline was 89.6% greater in the NAC group compared with the control group ($P = 0.09$).

Exploratory analysis of secondary efficacy showed that changes in exercise physiology, PFT parameters or serum markers of pneumocyte injury did not differ significantly between the NAC and control groups, except for the changes in % predicted VC in the same subsets of IPF patients. The mean decreases in % predicted VC in a subset of patients with initial FVC <95% of predicted were 1.8% and 4.9% in the NAC and control groups, respectively, with the difference being significant ($P = 0.025$) (Fig. 4a). Likewise, the decreases in % predicted VC in a subset of patients with initial DL_{CO} <55% of predicted were 2.8% and 5.9% in the NAC and control groups, respectively, with the difference being significant ($P = 0.019$) (Fig. 4b).

Safety

The severity of adverse events was assessed using the grading scale of the Common Terminology Criteria for Adverse Events v3.0 (National Cancer Institute, http://ctep.cancer.gov). Common adverse events reported during the study period were bacterial pneumonia, cough, sore throat and hypercholesterolaemia (Table 5). There were no significant differences in the number of adverse events reported for the two groups. The severity of these adverse events was less than grade 2 for the whole NAC group. Therefore, treatment with NAC was generally well tolerated.

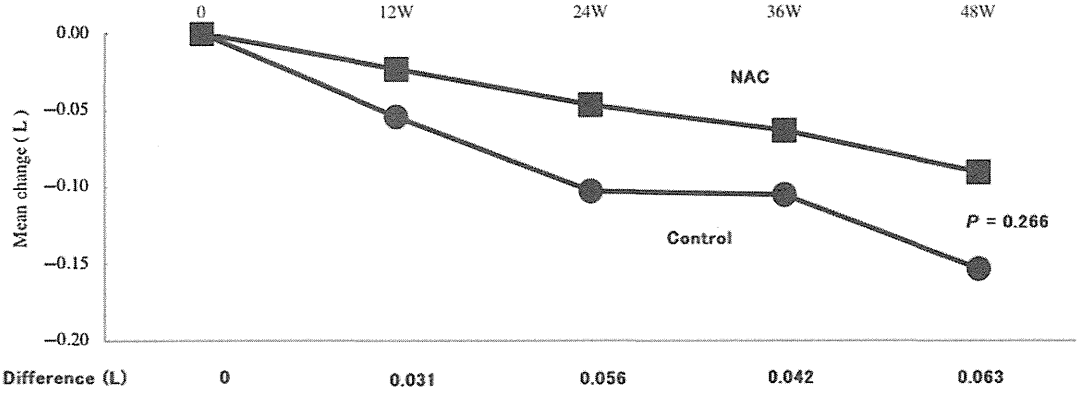
DISCUSSION

Acetylcysteine is a precursor of the antioxidant, GSH, the levels of which are reduced in the lungs of patients

Table 2 Clinical characteristics and demographical parameters for patients with idiopathic pulmonary fibrosis (IPF) who were treated or not treated with N-acetylcysteine

	All patients: completed study		
	NAC ($n = 38$)	Control ($n = 38$)	*P* value
Sex			
Male	29 (76%)	29 (76%)	1.00
Female	9 (24%)	9 (24%)	
Age, years (mean ± SD)	67.6 ± 6.4	68.2 ± 7.7	0.78
Smoking			
Current	3 (7.9%)	3 (7.9%)	1.00
Ex	25 (65.8%)	26 (68.4%)	
Never	10 (26.3%)	9 (23.7%)	
Years since IPF diagnosis (years)			
<1	9 (23.7%)	5 (13.2%)	0.09
1–3	17 (44.7%)	14 (36.8%)	
>3	12 (31.6%)	19 (50%)	
Mean ± SD, years	3.0 ± 3.4	3.2 ± 2.5	
Prior treatment with oral corticosteroids and NAC			
None	38 (100%)	38 (100%)	1.00
Disease severity stage	(I:30, II:8)	(I:31, II:7)	1.00
FVC, % predicted (mean ± SD)	89.2 ± 17.8	88.7 ± 15.5	0.56
VC, % predicted (mean ± SD)	90.4 ± 18.3	89.1 ± 15.0	0.95
TLC, % predicted (mean ± SD)	82.5 ± 17.4	81.2 ± 13.3	0.84
DL_{CO}, % predicted (mean ± SD)	72.3 ± 25.3	64.4 ± 20.1	0.16
Lowest Spo2 during a 6MWT, % (mean ± SD)	93.1 ± 2.1	92.4 ± 2.0	0.14
KL-6, U/mL (mean ± SD)	995.1 ± 440.0	1246.8 ± 114.9	0.78
SP-D, ng/mL (mean ± SD)	179.6 ± 102.7	203.4 ± 107.4	0.40

6MWT; 6-min walk test; DL_{CO}, diffusing capacity of carbon monoxide; FVC, forced vital capacity; NAC, N-acetylcysteine; SD, standard deviation; SP-D, surfactant protein D; TLC, total lung capacity; VC, vital capacity.



			0		12W		24W		36W		48W	ANCOVA
FVC(L)	NAC	n	38		38		38		38		38	P = 0.266
		FVC(L)(m ± SD)	2.76 ± 0.81		2.74 ± 0.79		2.71 ± 0.80		2.70 ± 0.81		2.67 ± 0.84	
		ΔFVC(m ± SD)			−0.02 ± 0.24		−0.05 ± 0.24		−0.06 ± 0.25		−0.09 ± 0.30	
	Control	n	38		38		38		38		38	
		FVC(L)(m ± SD)	2.66 ± 0.64		2.61 ± 0.66		2.56 ± 0.67		2.56 ± 0.68		2.51 ± 0.68	
		ΔFVC(m ± SD)			−0.05 ± 0.19		−0.10 ± 0.19		−0.11 ± 0.18		−0.15 ± 0.20	

Figure 2 The mean changes in forced vital capacity (FVC) at 48 weeks (W), as determined by analysis of covariance (ANCOVA), were decreases of 90 mL and 150 mL in patients in the N-acetylcysteine (NAC) and control groups, respectively ($P = 0.2661$). SD, standard deviation.

Table 3 Changes in high-resolution computed tomography findings in patients with idiopathic pulmonary fibrosis who were treated or not treated with N-acetylcysteine

	Findings	12 weeks	24 weeks	36 weeks	48 weeks	
N-acetylcysteine ($n = 38$)	Improvement, *n*	0	1	1	3	31/35(88.6%)
	Stable, *n*	35	28	32	28	
	Progression, *n*	0	6	2	4	4/35 (11.4%)
	Missing data, *n*	3	3	3	3	
Control ($n = 38$)	Improvement, *n*	0	0	0	0	25/32 (78.1%)
	Stable, *n*	31	27	30	25	
	Progression, *n*	1	5	2	7	7/32 (21.9%)
	Missing data, *n*	6	6	6	6	

$P = 0.33$ (48 weeks).

Table 4 Changes in subjective assessment of dyspnoea in patients with idiopathic pulmonary fibrosis who were treated or not treated with N-acetylcysteine

	Dyspnoea, *n*	12 weeks	24 weeks	36 weeks	48 weeks	
NAC ($n = 38$)	Improved	2	2	3	2	33 (86.8%)
	Stable	32	30	29	31	
	Deteriorated	4	6	6	5	5 (13.2%)
Control ($n = 38$)	Improved	0	1	1	1	32 (84.2%)
	Stable	37	34	33	31	
	Deteriorated	1	3	4	6	6 (15.8%)

$P = 1.00$ (48 weeks).

Table 5 Frequent adverse events in patients with idiopathic pulmonary fibrosis who were treated or not treated with N-acetylcysteine (full analysis set)

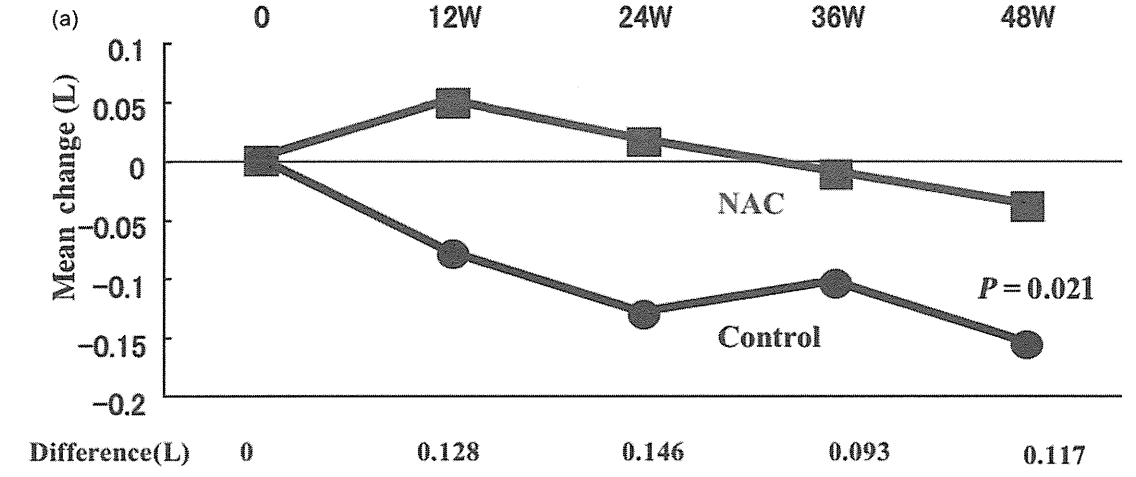
	NAC ($n = 44$)				Control ($n = 46$)			
Adverse event	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4
Bacterial pneumonia	2	2	0	0	0	0	0	0
Cough	1	1	0	0	0	0	0	0
Sore throat	2	0	0	0	0	0	0	0
Hypercholesteraemia	2	0	0	0	0	0	0	0

Grade 1, mild; Grade 2, moderate; Grade 3, severe; Grade 4, life-threatening or disabling; NAC, N-acetylcysteine.

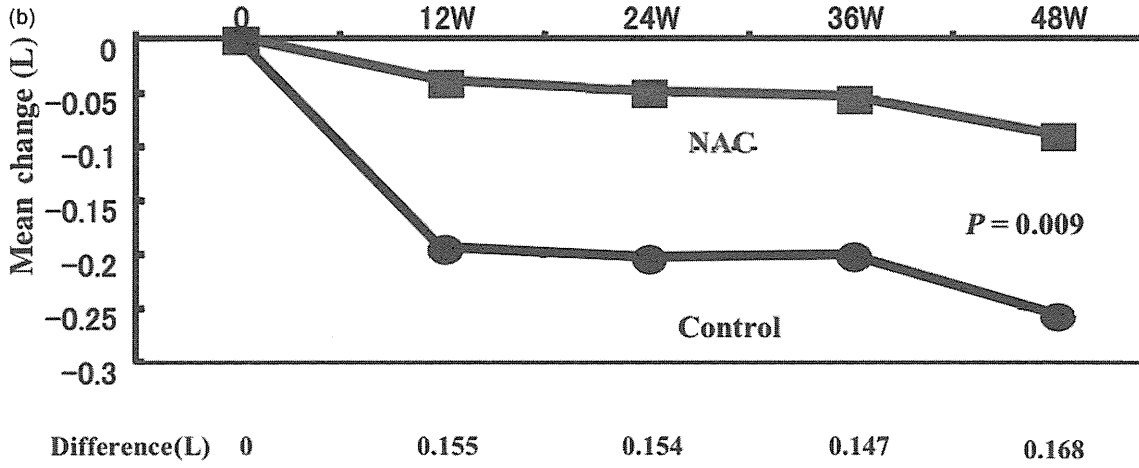
with IPF.[2,3] Administration of NAC is largely free of adverse effects, and it has been used for many years as a mucolytic agent, either by direct instillation or nebulization into the airways.

Aerosol administration of NAC, as used in this study, is a rational approach for delivery of a pharmacological dose to the lungs of patients with IPF. Hagiwara *et al.* showed that inhalation of NAC increased the neutralization of oxygen radicals in an animal model of bleomycin-induced lung fibrosis.[13] We have also shown that the clinical efficacy of NAC, when inhaled at the same dose as used in the present study, correlated with the improvement in redox imbalance in patients with IPF.[29] Moreover, Borok *et al.* demonstrated that aerosol therapy with GSH (600 mg twice daily for 3 days) was safe in patients with IPF and was biologically efficacious because it interacted favourably with both intracellular and extracellular events resulting in a net reduction in the oxidant burden at the alveolar epithelial surface.[4]

A placebo-controlled, randomized trial comparing the effect of high-dose oral acetylcysteine with that of placebo in patients receiving prednisone plus azathioprine has been completed.[10] In that study, the 12-month declines in VC and DL_{CO} were significantly less in the acetylcysteine-treated patients (VC: 0.18 L difference; DL_{CO}: 0.75 mmol/min/kPa difference). Moreover, the decline in VC in patients in the placebo arm was 190 mL, which is comparable with that reported for patients in the placebo arms of other studies. As all patients in that trial also received prednisone and azathioprine, there is ongoing discussion as to whether this treatment effect is seen only in patients receiving the combination of NAC with

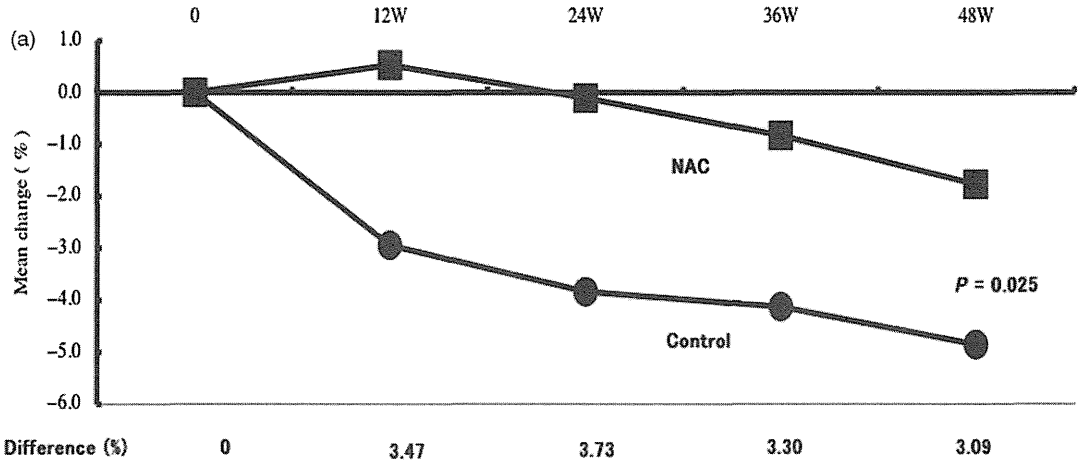


			0	12W	24W	36W	48W	ANCOVA
FVC(L)	NAC	n	24	24	24	24	24	P = 0.021
		FVC (L)(m ± SD)	2.36 ± 0.49	2.41 ± 0.60	2.38 ± 0.57	2.35 ± 0.55	2.32 ± 0.64	
		ΔFVC(m ± SD)		0.05 ± 0.24	0.02 ± 0.23	−0.01 ± 0.18	−0.04 ± 0.28	
	Control	n	25	25	25	25	25	
		FVC (L)(m ± SD)	2.45 ± 0.58	2.37 ± 0.60	2.32 ± 0.62	2.35 ± 0.65	2.29 ± 0.63	
		ΔFVC(m ± SD)		−0.08 ± 0.21	−0.13 ± 0.18	−0.10 ± 0.18	−0.16 ± 0.20	

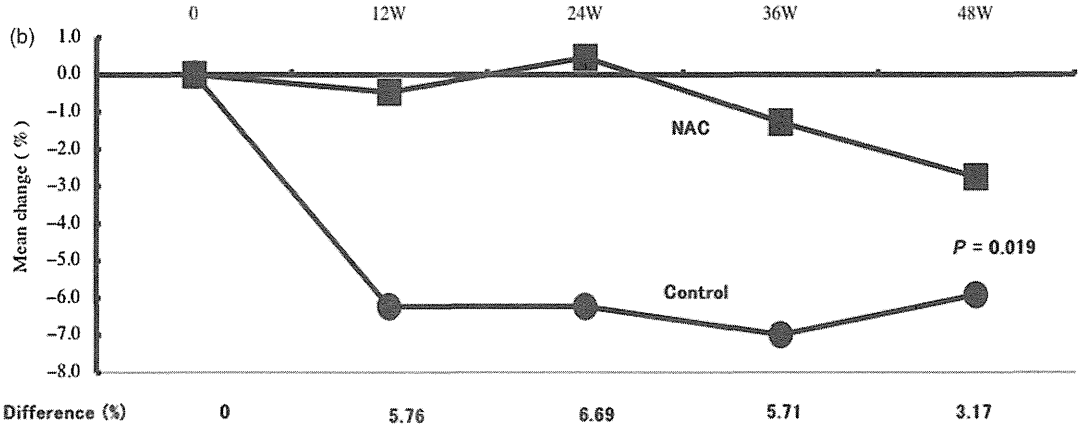


			0	12W	24W	36W	48W	ANCOVA
FVC(L)	NAC	n	8	8	8	8	8	P = 0.009
		FVC (L) (m ± SD)	2.51 ± 0.50	2.47 ± 0.42	2.46 ± 0.45	2.45 ± 0.47	2.42 ± 0.47	
		ΔFVC (m ± SD)		−0.04 ± 0.16	−0.05 ± 0.27	−0.06 ± 0.21	−0.09 ± 0.17	
	Control	n	13	13	13	13	13	
		FVC (L) (m ± SD)	2.29 ± 0.63	2.09 ± 0.55	2.08 ± 0.56	2.08 ± 0.59	2.03 ± 0.56	
		ΔFVC (m ± SD)		−0.19 ± 0.24	−0.20 ± 0.15	−0.20 ± 0.15	−0.26 ± 0.17	

Figure 3 (a) Exploratory analysis of primary efficacy showed that the mean changes in forced vital capacity (FVC) in a subset of patients with initial FVC <95 % of predicted were decreases of 40 mL and 160 mL in the N-acetylcysteine (NAC) and control groups, respectively ($P = 0.0213$). (b) The mean changes in FVC in a subset of patients with initial diffusing capacity of carbon monoxide <55% of predicted were decreases of 90 mL and 260 mL in the NAC and control groups, respectively ($P = 0.0086$). ANCOVA, analysis of covariance; m, mean; SD, standard deviation; W, weeks.



%VC(%)		0	12W	24W	36W	48W	ANCOVA
NAC	n	24	24	24	24	24	P = 0.025
	%VC(%)(m ± SD)	79.62 ± 11.06	80.16 ± 13.9	79.51 ± 13.94	78.79 ± 13.35	77.85 ± 15.11	
	Δ%VC(m ± SD)		0.53 ± 5.79	−0.11 ± 6.36	−0.83 ± 6.32	−1.77 ± 8.12	
Control	n	25	25	25	25	25	
	%VC(%)(m ± SD)	81.96 ± 10.83	79.02 ± 12.92	78.11 ± 14.34	77.8 ± 14.83	77.1 ± 13.78	
	Δ%VC(m ± SD)		−2.94 ± 6.18	−3.84 ± 6.71	−4.13 ± 7.28	−4.86 ± 6.36	



%VC(%)		0	12W	24W	36W	48W	ANCOVA
NAC	n	8	8	8	8	8	P = 0.019
	%VC(%)(m ± SD)	82.58 ± 9.9	82.11 ± 9.65	83.05 ± 10.86	81.3 ± 10.07	79.83 ± 10.58	
	Δ%VC(m ± SD)		−0.47 ± 3.21	0.46 ± 6.77	−1.28 ± 6.76	−2.75 ± 6.21	
Control	n	13	13	13	13	13	
	%VC(%)(m ± SD)	79.90 ± 12.44	73.66 ± 13.71	73.66 ± 14.22	72.9 ± 14.17	73.97 ± 12.72	
	Δ%VC(m ± SD)		−6.23 ± 6.82	−6.23 ± 4.95	−6.99 ± 5.38	−5.92 ± 5.47	

Figure 4 (a) The mean changes in % predicted vital capacity (VC) in a subset of patients with initial forced VC (FVC) <95% of predicted were decreases of 1.8% and 4.9% in the N-acetylcysteine (NAC) and control groups, respectively ($P = 0.0245$). (b) The mean changes in % predicted VC in a subset of patients with initial diffusing capacity of carbon monoxide <55% of predicted were decreases of 2.8% and 5.9% in the NAC and control groups, respectively ($P = 0.0188$). ANCOVA, analysis of covariance; m, mean; SD, standard deviation; W, weeks.

prednisone and azathioprine, or whether NAC alone is responsible for this effect. The limitations of that study included the lack of a true 'no-therapy' arm. In order to answer this question, the National Institutes of Health began enrolment for the PANTHER-IPF (Prednisone, Azathioprine, and N-acetylcysteine: A study that Evaluates Response in Idiopathic Pulmonary Fibrosis) trial in 2009. This double-blind, three-arm trial was designed to evaluate the efficacy of NAC alone or NAC combined with prednisone and

Table 6 Trials of N-acetylcysteine therapy in patients with idiopathic pulmonary fibrosis

Study	Baseline PFT (mean)	Study design	Therapeutic agents	Dose	Completed No.	Rx duration	Primary endpoints	Secondary endpoints	Results (absolute difference in VC or FVC)
				Randomized controlled trials					
Demedts *et al.*, 2005[10]	%VC: 65%	DB, PRCT	Aza/Pred/NAC	2 mg/kg; 0.5 mg/kg-10 mg; 600 mg tid 2 mg/kg; 0.5 mg/kg-10 mg	57	12 months	VC	DL_{CO}/VA, CRP, dyspnoea CPET, HRCT, SGRQ AE, survival	Less deterioration in **VC and DL_{CO}** with addition of NAC (0.18 L)
	%DL_{CO}: 43%		Aza/Pred/Placebo		51		DL_{CO}		
Tomioka *et al.*, 2005[31]	%VC: 68%	Pilot RCT	NAC	176 mg nebulized bid	10	12 months	VC	6MWT, HRCT KL-6, SF-36	No difference in VC and DL_{CO}, improved change in **SaO2 during 6MWT and KL-6** with NAC, worse HRCT with control
	%DLco: 65%		Bromhexine	2 mg nebulized bid	12		DL_{CO}		
Homma *et al.* (this study) 2012	**%VC: 90%**	RCT	NAC	352.4 mg nebulized bid	38	48 weeks	FVC	6MWT, VC, %VC, TLC, %TLC, DL_{CO}, %DL_{CO}, Dyspnoea, HRCT	Less deterioration in **FVC and %VC** in a subset group (mean baseline %VC: 80%, %DL_{CO}: 43%) with NAC (0.12–0.17 L)
	%DL_{CO}: 72%		Control (none)	No therapy	38				
				Prospective, nonrandomized trials					
Behr *et al.*, 1997[7]	%VC: 81%	OLT	NAC	600 mg tid	20 (10 IPF, 10 CTD)	12 weeks	BAL, Oxidative stress	VC, TLC, DL_{CO}, PaO_2	Increased total **glutathione and reduced glutathione**; decreased spontaneous oxidative activity Improved change in **lung function index**
	%DL_{CO}: 57%								

6MWT, 6-min walk test; AE, acute exacerbation; Aza, azathioprine; CPET, cardiopulmonary exercise testing; CRP, C-reactive protein; CTD, connective tissue disease; DB, double-blinded; DL_{CO}, diffusing capacity of carbon monoxide; HRCT, high-resolution computed tomography; IPF, idiopathic pulmonary fibrosis; KL-6, Krebs von den Lungen-6 ; NAC, N-acetylcysteine; PaO_2, partial arterial oxygen concentration; PFT, pulmonary function test; PRCT, placebo-controlled randomized trial; Pred, prednisone; OLT, open-label trial; RCT, randomized controlled trial; SaO_2, arterial oxygen saturation; SF-36, Short Form 36 Health Survey; SGRQ, St. George's Respiratory Questionairre; TLC, total lung capacity; VC, vital capacity.

azathioprine compared with a placebo control group using the decline in FVC as the primary endpoint.[30]

Another previous pilot study randomized 22 patients to receive aerosolized NAC (at half the dose used in the present study) or placebo for 12 months; significant improvements were documented in the lowest arterial oxygen saturation during a 6MWD test, the extent of ground-glass opacity on CT and the reduction in KL-6 levels, but there were no differences in lung function indices. The dose of NAC used in that study (352.4 mg per day) may have been too low for effective therapy in patients with IPF.[31]

In one prospective, nonrandomized study, 20 patients with IPF or fibrosing alveolitis due to connective tissue disease were treated with oral NAC for 12 weeks and showed an increase in GSH concentrations, a decrease in products of oxidative stress and improvements in lung function (Table 6).

Moreover, these trials demonstrated that baseline % predicted VC was 65–81% and % predicted DL_{CO} was 43–65% in the patients in whom therapy was effective. Therefore, we identified two subsets of patients for the post hoc exploratory analyses: the first with a mean baseline VC of almost 80% of predicted corresponding to the subgroup with initial FVC <95% of predicted (baseline % predicted VC: 79.6 ± 11.1% in the NAC group vs 82.0 ± 10.8% in the control group) and the second subset with a mean baseline DL_{CO} of almost 43% of predicted, corresponding to the subgroup with initial DL_{CO} <55% of predicted (baseline % predicted DL_{CO}: 41.7 ± 10.2% in the NAC group vs 42.6 ± 7.1% in the control group).

In the present study, which included a true 'no treatment' arm, NAC monotherapy in a subset of patients with a mean baseline VC of almost 80% of predicted and a mean DL_{CO} of almost 43% of predicted resulted in significantly smaller 48-week declines in FVC (a difference of 120–170 mL). Beeh *et al.* reported a positive correlation between sputum GSH concentrations and lung function.[32] Therefore, it may be reasonable to replenish GSH levels in these advanced stages even in patients with early stage IPF.

It has recently been shown that decreases in FVC of 10% or more from baseline over a period of 6–12 months are associated with an increased risk of a poor prognosis in patients with IPF.[33–37] In the present overall and subgroup analyses of the incidence of serial decline in FVC at 48 weeks, the percentage of patients with a >10% decline in FVC was 36.4–76.8% lower, and the percentage of patients with a <10% decline in FVC was 14.8–89.6% greater in the NAC group than in the control group. These results imply that NAC therapy may improve the prognosis of patients with IPF.

The present study has several limitations in that it was not placebo-controlled, double-blind or designed to assess survival. In addition, the dropout rate of 24% during the study limited the robustness of the results.

Because the present study demonstrated positive results in some patients treated with inhaled NAC, without the use of any immunosuppressive or antifibrotic agents, we anticipate that NAC will be a candidate drug for IPF therapy in the future. Further trials of NAC alone versus pirfenidone alone and NAC combined with prednisone and azathioprine or pirfenidone will be needed to guide the development of new therapeutic strategies for patients with IPF in the future.

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Original Article

Newly Established Monoclonal Antibodies for Immunological Detection of H5N1 Influenza Virus

Kazuo Ohnishi[1], Yoshimasa Takahashi[1], Naoko Kono[2], Noriko Nakajima[3], Fuminori Mizukoshi[1], Shuhei Misawa[4], Takuya Yamamoto[1], Yu-ya Mitsuki[1], Shu-ichi Fu[1], Nakami Hirayama[1], Masamichi Ohshima[1], Manabu Ato[1], Tsutomu Kageyama[2], Takato Odagiri[2], Masato Tashiro[2], Kazuo Kobayashi[1], Shigeyuki Itamura[2], and Yasuko Tsunetsugu-Yokota[1]*

[1]Department of Immunology, [2]Influenza Virus Research Center, and [3]Department of Pathology, National Institute of Infectious Diseases, Tokyo 162-8640; and [4]Tsuruga Institute of Biotechnology, Toyobo, Co., Ltd., Fukui 914-8550, Japan

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SUMMARY: The H5N1 subtype of the highly pathogenic (HP) avian influenza virus has been recognized for its ability to cause serious pandemics among humans. In the present study, new monoclonal antibodies (mAbs) against viral proteins were established for the immunological detection of H5N1 influenza virus for research and diagnostic purposes. B-cell hybridomas were generated from mice that had been hyperimmunized with purified A/Vietnam/1194/2004 (NIBRG-14) virion that had been inactivated by UV-irradiation or formaldehyde. After screening over 4,000 hybridomas, eight H5N1-specific clones were selected. Six were specific for hemagglutinin (HA) and had in vitro neutralization activity. Of these, four were able to broadly detect all tested clades of the H5N1 strains. Five HA-specific mAbs detected denatured HA epitope(s) in Western blot analysis, and two detected HP influenza virus by immunofluorescence and immunohistochemistry. A highly sensitive antigen-capture sandwich ELISA system was established by combining mAbs with different specificities. In conclusion, these mAbs may be useful for rapid and specific diagnosis of H5N1 influenza. Therapeutically, they may have a role in antibody-based treatment of the disease.

INTRODUCTION

The highly pathogenic (HP) H5N1 avian influenza virus caused the first outbreak in humans in Hong Kong in 1997. This outbreak resulted in the infection of 18 people and resulted in six deaths (1,2). Thereafter, it was determined that H5N1 avian influenza virus was continuously circulated among geese in Southeastern China. Eventually, it spread to other Southeast Asian countries, where it severely damaged poultry farms (3,4). Subsequent H5N1 outbreaks in humans occurred in China and Vietnam in 2003 and in Indonesia in 2005. The most recent endemic has occurred in Egypt. According to a World Health Organization report, the H5N1 avian influenza virus had infected 565 people and resulted in 331 deaths by August 19, 2011 (5). Therefore, although sporadic, this fatal human infection is persistent and has the potential to cause serious future pandemics.

In humans, infection with HP H5N1 avian influenza virus causes high fever, coughing, shortness of breath, and radiological findings of pneumonia (6–8). In severe cases, rapidly progressive bilateral pneumonia develops, causing respiratory failure and may be responsible for the high mortality associated with this virus. de Jong et al. analyzed human cases of H5N1 infection and found that a high viral load and the resulting intense inflammatory response caused severe symptoms; furthermore, viral RNA was frequently detected in the rectum, blood, and nasopharynx (9). Thus, it is essential to detect HP influenza virus infection early and rapidly in order to provide early interventions that protect patients from devastating respiratory failure that arises from a high viral load. Additionally, early viral detection would facilitate rapid identification of infected patients and prevent unregulated contact with other people.

The present diagnostic standard for HP H5N1 influenza is the presence of the neutralization antibody. However, it takes more than 1 week for H5N1-specific antibodies to develop, and a well-equipped biosafety level 3 (BSL3) laboratory is required for the virus neutralization assay. A simpler method is the hemagglutination-inhibition assay using horse erythrocyte. This method has been widely performed on paired acute and convalescent sera from patients with HP H5N1 influenza virus infections. Although this method has acceptable sensitivity, its specificity has been questioned (7).

Isolating the virus from patient samples is the gold standard for diagnosing an infection; however, this is not always possible. For example, the method of sample preparation and preservation strongly influence the ability to isolate the virus. Moreover, a BSL3 laboratory is essential. At present, the most sensitive and rapid method for initial diagnosis of H5N1 virus infections is by conventional or real-time reverse-transcriptase polymerase chain reaction (RT-PCR). However, this proce-

*Corresponding author: Mailing address: Department of Immunology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111, Fax: +81-5285-1150, E-mail: yyokota@nih.go.jp

dure requires expertise in molecular virology and expensive equipment and reagents. Moreover, because of its high sequence specificity, this approach could fail to identify mutant influenza viruses that continually evolve due to a high mutation rate (8).

For screening suspected H5N1 influenza virus in the field, the ideal approach would be to employ an immunology-based technique that detects viral antigens. Such a method is simple and rapid. However, its sensitivity and specificity depend highly on the antibodies used. Thus, an immunological assay that uses appropriate specific antibodies against H5N1 in combination with specific antibodies against other subtypes of influenza virus or viruses that cause febrile diseases would be useful for screening in areas with endemic influenza-like illness. While there are several rapid influenza virus diagnostic systems available for seasonal influenza (10), few exist for H5N1 influenza. Therefore, we have developed a simple and rapid diagnostic system with high sensitivity and specificity for H5N1 influenza virus.

Influenza virus belongs to the family *Orthomyxoviridae*; its genome consists of a negative-sense, single-stranded RNA with eight segments, each encoding structural and non-structural proteins (11). Influenza A viruses are classified into several subtypes based on the hemagglutinin (HA) and neuraminidase (NA) serotypes. In total, there are 16 HA and 9 NA serotypes. The H5N1 viruses are divided into clades 1 and 2 based on their HA genotypes. Clade 2 has been further subdivided into five sub-clades (12). Clade 1 viruses were predominant in Vietnam, Thailand, and Cambodia in the early phase of the 2004–2005 outbreak, whereas clade 2.1 viruses were endemic in Indonesia at that time (8). These two viruses are the major prototypes for the preparation of prepandemic H5N1 vaccines. We used inactivated purified clade 1 virion [A/Vietnam/1194/2004 (NIBRG-14)] as an immunizing antigen to establish mouse monoclonal antibodies (mAbs) specific for H5N1 influenza virus. Characterization of these mAbs revealed that they could detect H5N1 viruses when used in an immunofluorescence staining assay (IFA), Western blotting analysis, immunohistochemistry, and antigen-capture sandwich ELISA. In addition, the mAbs had significant in vitro neutralization activity against H5N1 viruses, and some broadly detected both clade 1 and 2 viruses.

MATERIALS AND METHODS

Viruses and cell culture: The NIBRG-14 (H5N1) virus, which possesses modified HA and NA genes derived from the A/Vietnam/1194/2004 strain on the backbone of six internal genes of A/Puerto Rico/8/34 (PR8), was provided by the National Institute for Biological Standards and Controls (NIBSC; Potters Bar, UK). A/Indonesia/05/2005 (Indo5/PR-8-RG2), A/Turkey/1/2005 (NIBRG-23), A/Anhui/01/2005 (Anhui01/PR8-RG5) were also obtained from NIBSC. All non-H5N1 strains were obtained from a stockpile of seed vaccines of the Influenza Virus Research Center of the National Institute of Infectious Diseases. The live virus was manipulated in a BSL2 laboratory. To produce and purify the virion, the NIBRG-14 and PR8 viruses were propagated in the allantoic cavity of 10-day-old embryonated hens' eggs and purified through a 10–50% discontinuous sucrose gradient by ultracentrifugation (13). The viruses were then resuspended in phosphate-buffered saline (PBS) and inactivated by ultraviolet (UV) irradiation or by treatment with 0.05% formalin at 4°C for 2 weeks. These preparations were served as the inactivated H5N1 virus fraction. These conditions have been previously shown to completely inactivate H5N1 viruses.

Production of mAbs: Nine-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) were immunized subcutaneously with 20 μg of UV- or formaldehyde-inactivated NIBRG-14 (H5N1) virus using Freund's Complete Adjuvant (Sigma, St. Louis, Mo., USA). Two weeks later, the mice were boosted with a subcutaneous injection of 5 μg of the inactivated virus emulsified with Freund's Incomplete Adjuvant (Sigma). Three days after the boost, sera from the mice were tested by ELISA to determine the antibody titer against the NIBRG-14 virus. The three mice with the highest antibody titers were given an additional boost 14 days after the first boost by intravenous injection of 5 μg of the inactivated virus. Three days later, the spleens of these three mice were excised, and the spleen cells were fused with Sp2/O-Ag14 myeloma cells using the polyethylene glycol method of Kozbor and Roder (14). The fused cells were cultured on twenty 96-well plates and selected with hypoxiantine-aminopterin-thymidine (HAT) medium. The first screening was conducted by ELISA using formalin-inactivated purified NIBRG-14 (H5N1) and PR-8 (H1N1) virions, which were lysed with 1% Triton X100. The lysates (1 mg/ml) were diluted 2,000-fold with ELISA-coating buffer (50 mM sodium bicarbonate, pH 9.6), and the ELISA plates (Dynatech, Chantily, Va., USA) were coated at 4°C overnight. After blocking with 1% ovalbumin in PBS-Tween (10 mM phosphate buffer, 140 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h, the culture supernatants of the HAT-selected hybridomas were added and incubated for 1 h. After washing with PBS-Tween, the bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse IgG (1:2,000; Zymed, South San Francisco, Calif., USA) and *p*-nitrophenyl phosphate, which served as a substrate. In this first screening, hybridomas that reacted to the H5N1 virus (NIBRG-14) but not to the H1N1 virus (PR-8) were selected.

Baculoviral expression of recombinant HA and NA: Recombinant HA (rHA) and NA (rNA) proteins were produced as previously described (13). Briefly, the HA- and NA-coding genes of NIBRG-14 were amplified by PCR to attach a 6x-His tag to the C terminus of HA and to the N terminus of NA. The amplified DNAs were then cloned into pBacPAK8 (Clontech, Mountain View, Calif., USA) and transfected into Sf-21 (*Spodoptera frugiperda*) insect cells. Recombinant baculoviruses containing the rHA and rNA genes were isolated were used to infect Sf-21 cells. The recombinant proteins tagged with 6x-His were purified with TALON columns (Clontech) according to the manufacturer's protocol.

Neutralization assay: For the neutralization assay, 100 $TCID_{50}$ of H5N1 virus, a standard tissue culture infectious dose for such assays, was incubated for 30 min at 37°C in the presence or absence of the purified mAbs, which had been serially diluted twofold. The viruses

were then added to MDCK cell cultures that had been grown to confluence in a 96-well microtiter plate. The virus strains used were A/Vietnam/1194/2004 (NIBRG-14) (H5N1) (clade 1), A/Indonesia/05/2005 (Ind05/PR8-RG2) (H5N1) (clade 2.1), A/Turkey/1/2005 (NIBRG-23) (H5N1) (clade 2.2), and A/Anhui/01/2005 (Anhui01/PR8-RG5) (H5N1) (clade 2.3). After 3–5 days, the cells were fixed with 10% formaldehyde and stained with crystal violet to visualize the cytopathic effects induced by the virus (15). Neutralization antibody titers were expressed as the minimum concentration of purified immunoglobulin that inhibited a cytopathic effect.

Western blot analysis: UV-inactivated purified H5N1 virus (0.5 μg/lane) was loaded on SDS-PAGE gels under reducing conditions. The proteins were then transferred to a PVDF membrane (Genetics, Tokyo, Japan). After blocking with BlockAce reagent (Snow Brand Milk Products Co., Tokyo, Japan), the membranes were detected with the mAbs or diluted sera (1:1,000) that had been obtained from mice immunized with UV-irradiated H5N1 virus. After washing, the membrane was reacted with the peroxidase-conjugated $F(ab')_2$ fragment of anti-mouse IgG (H+L) (1:20,000; Jackson ImmunoResearch, West Grove, Pa., USA), and the bands were visualized on X-ray film (Kodak, Rochester, N.Y., USA) with chemiluminescent reagents (Amersham Biosciences, Piscataway, N.J., USA).

Purification and biotinylation of mAbs: Hybridomas were grown in Hybridoma-SFM medium (Invitrogen, Carlsbad, Calif., USA) supplemented with recombinant IL-6, penicillin (100 U/mL), and streptomycin (100 μg/mL) (16). The culture supernatants were harvested, and 1/100 volume of 1 M Tris-HCl (pH 7.4) and 1/500 volume of 10% NaN_3 were applied directly on a Protein G-Sepharose 6B column (Amersham Biosciences). The column was washed with PBS and eluted with glycine/HCl (pH 2.8). After measuring the OD_{280} of the fractions, the protein-containing fractions were pooled, and an equal volume of saturated $(NH_4)_2SO_4$ was added. The precipitated proteins were dissolved in PBS, dialyzed against PBS, and stored at −20°C. The purified antibodies were biotinylated with sulfo-NHS-LC-biotin (Pierce, Rockford, Ill., USA) according to the manufacturer's protocol.

Antigen-capture ELISA: The purified antigen-capturing mAb was immobilized on a microplate (Immulon 2; Dynatech) by incubating 4 μg/mL of the mAb in 50 mM sodium bicarbonate buffer (pH 8.6) at 4°C overnight. The microplate was blocked with 1% BSA, washed with PBS-Tween, and reacted with serial dilutions of UV-inactivated purified H5N1 virus for 1 h at room temperature. After washing with PBS-Tween, biotinylated probing mAb (0.1 μg/mL) was added to the wells for 1 h at room temperature. After washing, horseradish peroxidase (HRP)-labeled streptavidin (Zymed) was added to the wells for 1 h at room temperature. After washing, 0.4 mg/mL *o*-phenylenediamine (OPD Sigma P-8412) in OPD Buffer (0.05 M citrate-phosphate buffer pH 5.0, 0.04% H_2O_2) or TMB(+) substrate (DAKO, Kyoto, Japan) was added. The reaction was stopped by adding 2N H_2SO_4, and the OD_{490} or OD_{450} was measured using a multi-well plate reader (Flow Laboratories Inc., Inglewood, Calif., USA).

Immunohistochemistry: Lung tissues were harvested from mice infected with A/Vietnam/1194/2004 (NIBRG-14) or A/HongKong/483/97 (HK483). In addition, autopsied lung tissues of patients infected with influenza virus (H1N1 or 2009 H1N1pdm) were used. Formaldehyde- or formalin-fixed paraffin-embedded lung tissue sections were deparaffinized with xylene and graded ethanol and then autoclaved in 0.1 M citrate-buffer (pH 6.0) at 121°C for 10 min to retrieve the antigens. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide for 30 min at room temperature. After blocking with M.O.M. blocking reagent (Vector laboratories, Burlingame, Calif., USA) or 5% goat serum, the sections were incubated with each of the mouse mAbs or rabbit polyclonal antibody against type A influenza nucleoprotein at 4°C overnight. After washing off the excess antibodies, the sections were incubated with HRP-labeled anti-mouse IgG followed by tyramide signal amplification system (Biotin-free catalyzed amplification system, CSAII; DAKO) or biotinylated anti-rabbit IgG followed by streptavidin/HRP (LSAB kit; DAKO). The labeled peroxidase activity was detected using diaminobenzidine (DAB; Dojin, Kumamoto, Japan) in 0.015% hydrogen peroxide/0.05 M Tris-HCl (pH 7.6). The sections were counterstained with hematoxylin.

RESULTS

Generation of H5N1-specific mAbs: To establish hybridomas that secrete mAbs specific for the H5N1 virus, BALB/c mice were immunized with the whole virion fraction of purified A/Vietnam/1194/2004 (NIBRG-14) virus. The virus had been inactivated by conventional formaldehyde-fixation or by UV-irradiation to avoid possible changes in antigenicity caused by aldehyde fixation. A standard immunization protocol was used, where mice were boosted twice at 2-week intervals with antigen emulsified first in Freund's Complete Adjuvant and then in Freund's Incomplete Adjuvant. Three days after the final boost, a cell suspension was prepared from the spleens of three immunized mice and fused with SP-2/O myeloma using a polyethylene-glycol method. The fused cells were then selected with HAT (14). Hybridoma screening yielded eight hybridoma clones that reacted to NIBRG-14 lysate but not PR-8 lysate in ELISA (Table 1). Of these clones, seven were from mice immunized with UV-inactivated virion, and one was from mice immunized with formaldehyde-inactivated virion. Six clones (Niid_H5A, Niid_H5B, Niid_H5C, Niid_H5D, Niid_H5E, and Niid_H5F) reacted to rHA protein from a H5N1 virus (recHA_H5N1), while one clone (Niid_N1A) reacted to rNA protein from a H5N1 virus (recNA_H5N1). The remaining clone (Niid_150KA) did not react to either recHA_H5N1 or recNA_H5N1 by ELISA but did react to a 150-kDa molecule on Western blot analysis (described below). Interestingly, seven of the eight clones were from the mice immunized with UV-inactivated virus. The eight hybridomas were successfully cloned by a repeated limiting-dilution method and adapted to a serum-free hybridoma culture medium. The purified antibodies from each clone were biotinylated and used for further experiments.

Table 1. Summary of the eight H5N1-specific mAbs generated in this study

Clone name	Old name	Ig-subclass	ELISA				Western blot	IFA	Histology	Neutralization (μg/mL)	Hemagglutination inhibition
			H5N1_ NIBRG-14	H1N1_ PR-8	recHA_ H5N1	recNA_ H5N1					
Niid_H5A[1)]	YH-1A1	IgG2a	+++	−	+	−	57 kDa	++		1.5 (Clade-dep)	−
Niid_H5B[1)]	YH-2F11	IgG2a	+++	−	+++	−	57 kDa			25	+
Niid_H5C[1)]	OM-A	IgG2a	+++	−	++	−	57 kDa		+(mo/hu)	12	
Niid_H5D[1)]	OM-B	IgG2a	+++	−	++	−	57 kDa		+(mo)	12	
Niid_H5E[1)]	OM-C	IgG2a	+++	−	++	−	57 kDa			12 (Clade-dep)	
Niid_H5F	AY-2C2	IgG1	+++	−	++	−	ND	++		6	−
Niid_N1A[1)]	YH-2D3	IgG2a	+++	−	−	+	ND	++			
Niid_150KA[1)]	OM-D	IgG1	+++	−	−	−	150 kDa	++			−

[1)]: Clones derived from mice immunized with UV-inactivated virus. The remaining clone is derived from a mouse immunized with formaldehyde-inactivated virus.

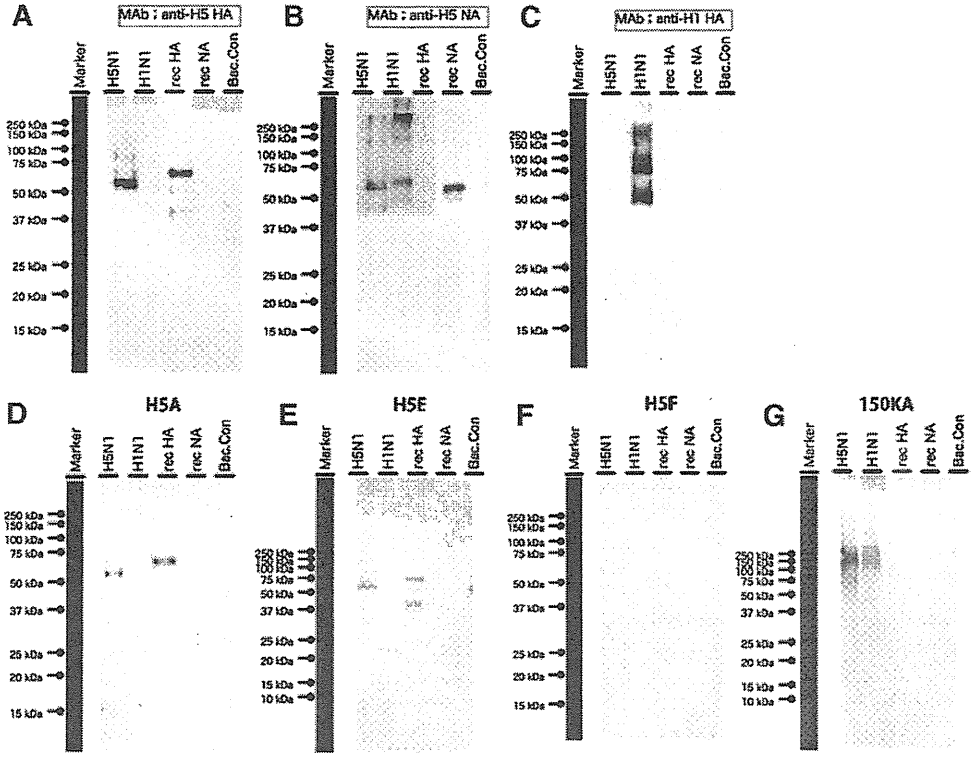

Fig. 1. Detection of influenza virus proteins in Western-blot analysis. Purified influenza virus proteins (0.5 μg/lane) were subjected to SDS-PAGE under reducing conditions. After blotting on a PVDF membrane, the proteins were detected by incubation with the eight monoclonal antibodies (mAbs), followed by incubation with the peroxidase-labeled $F(ab')_2$ fragment of donkey anti-mouse IgG. The mAbs were then visualized by chemiluminescent reaction. A, authentic anti-H5_hemagglutinin mAb; B, authentic anti-H5_neuraminidase mAb; C, authentic anti-H1_hemagglutinin mAb; D, Niid_H5A; E, Niid_H5E; F, Niid_H5F; G, Niid_150KA. The molecular weight markers are shown on the left.

Western blot analyses with the mAbs: Five mAbs (Niid_H5A, Niid_H5B, Niid_H5C, Niid_H5D, Niid_H5E) detected the 57-kDa H5_H1 protein by Western blot analysis, which suggests that the antibodies detected the linear epitope(s) of a HA1 fragment of H5_HA (Table 1 and Fig. 1). These antibodies also detected the 60-kDa recombinant H5-HA containing the His-tag. One of these clones, Niid_H5E, detected a 40-kDa subfragment of recombinant HA1, which suggests that the antigenic footprint detected by the mAb differs from that of the other four clones (Fig. 1). Niid-H5F, which reacted strongly to NIBRG-14 and rHA (H5) in ELISA, did not react to any proteins by Western blot analysis, presumably because the mAb detects a conformational epitope of H5-HA. The remaining clone, Niid_150KA, detected an unknown high molecular weight protein of approximately 150 kDa.

IFA with mAbs: Upon IFA, the HA-specific mAbs Niid-H5A and Niid_H5F, the NA-specific mAb Niid-N1A, and the Niid_150KA mAb that detects an

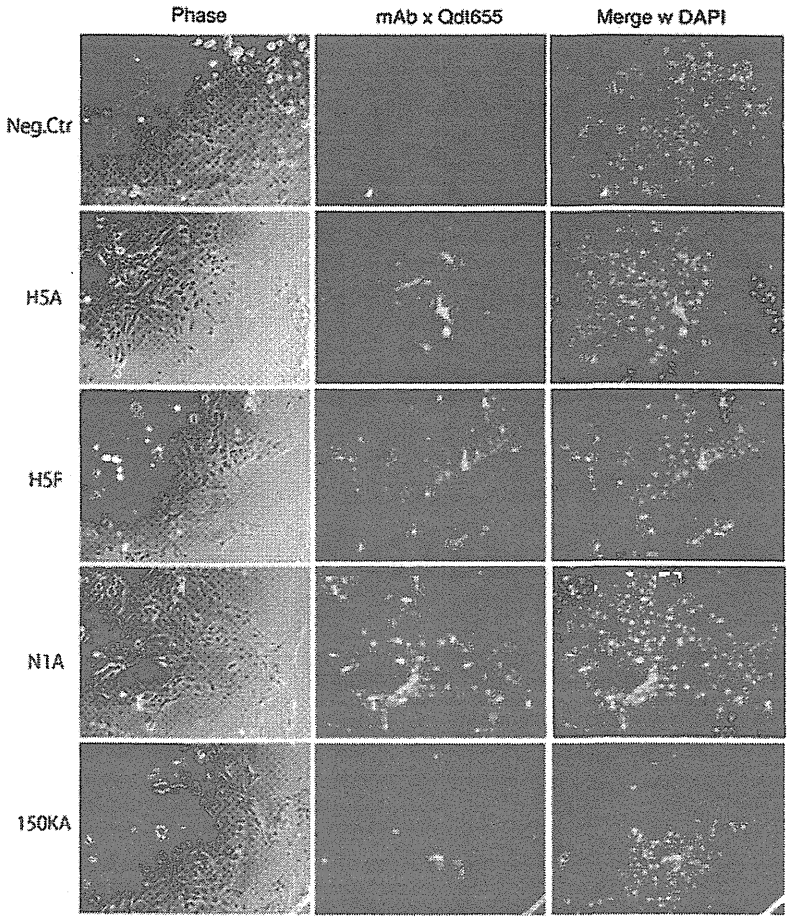

Fig. 2. Fluorescent immunostaining of H5N1 virus-infected MDCK cells with monoclonal antibodies (mAbs). Paraformaldehyde-fixed, H5N1 virus-infected MDCK cells were permeabilized by TBS-Tween and incubated with biotinylated mAbs. The mAbs were detected with Qdot655-conjugated streptavidin (red). Shown are representative staining patterns with Niid_H5A, Niid_H5F, Niid_N1A, and Niid_150KA. The negative control staining without mAb is shown on top. The nuclei were counterstained with DAPI (blue).

unknown 150-kDa protein bound to NIBRG-14-infected MDCK cells (Fig. 2). With the exception of Niid_H5F, these mAbs detected both the perinuclear region and the cell surface of NIBRG-14-infected MDCK cells. Niid_H5F did not detect the perinuclear region (presumably the Golgi body), which suggests that the antigenic footprint detected by this mAb differs from those of the other mAbs.

Immunohistochemistry: The Niid_H5C and Niid_H5D mAbs detected influenza virus antigens in the epithelial cells of the bronchioles and alveoli of 4% formaldehyde-fixed, paraffin-embedded lung tissue sections from mice infected with A/Vietnam/1194/2004 (NIBRG-14) (Fig. 3a). However, none of the mAbs detected influenza virus antigen in lung tissue sections from mice infected with A/HongKong/483/97 (HK483) (Fig. 3). In contrast, a polyclonal antibody against type A influenza nucleoprotein detected type A influenza virus nucleoprotein in the tissue sections from both the NIBRG-14- and HK483-infected mice (Fig. 3b, d). Thus, Niid_H5C and Niid_H5D specifically detected the HA antigen of A/Vietnam/1194/2004 (NIBRG-14). The specificity of these mAbs was then examined by using autopsied lung tissue sections from patients infected with seasonal influenza virus (H1N1) or 2009 pandemic influenza virus (2009H1N1pdm). Niid_H5C did not exhibit any crossreactivity, but the Niid_H5D mAb did show non-specific staining with the human lung section. Two other mAbs, Niid_H5B and Niid_N1A, were also subjected to such immunohistochemical analysis but did not show any reaction.

Neutralization assay with mAbs: The ability of the mAbs to neutralize several H5N1 influenza strains was then tested (Table 2). The four purified H5N1 virus strains, NIBRG-14, Indo-RG2, NIBRG-23, and Anhui-RG5, were diluted to 2–3 $\times$ 10^2 $TCID_{50}$/0.05 mL (Table 2, lower panel) and incubated with titrated amounts of anti-H5_HA mAbs. The remaining infectivity was then noted (Table 2, upper panel). Niid_H5A most potently neutralized the NIBRG-14 strain; it completely neutralized influenza virus infectivity at a concentration of 78 ng/mL. However, Niid_H5A was less potent in neutralizing the Indo-RG2 and Anhui-RG5 strains, which indicates that the neutralizing ability of this mAb was clade-dependent. In contrast, Niid_H5F and Niid_H5D exhibited relatively broad neutralizing abilities, since they neutralized all of the strains that were tested. Niid_H5C and Niid_H5E also showed characteristic clade-