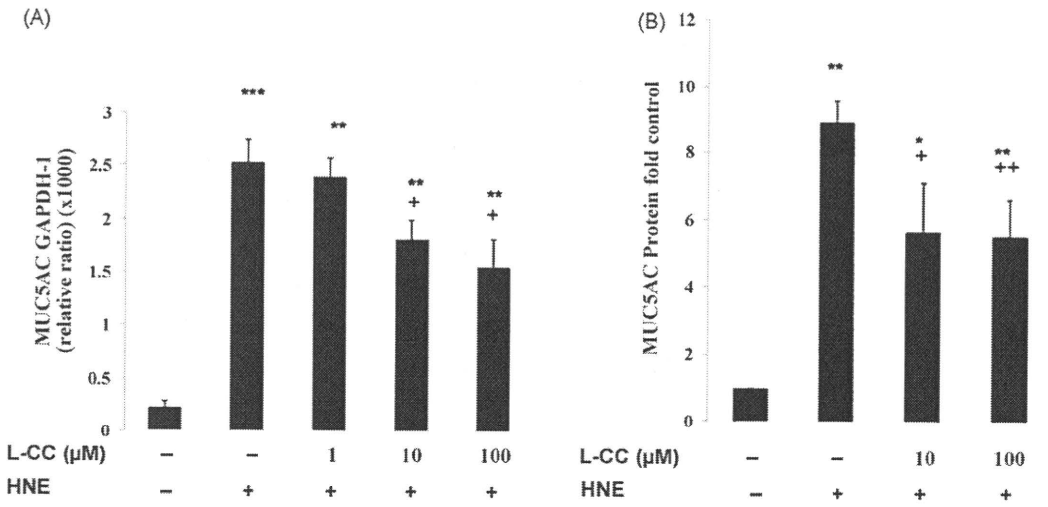

Fig. 1. MUC5AC mRNA expression in NCI-H292 cells (A) and MUC5AC protein concentration in supernatants (B) 30 min after stimulation with HNE in the presence of L-carbocisteine (L-CC) or vehicle (PBS). Results are mean ± S.E.M. from 6 samples (A) and from 8 samples (B). Significant differences from the values exposure to vehicle [control, L-CC (−) and HNE (−)] (*$P<0.05$, **$P<0.01$, and ***$P<0.001$), and from the values exposure to HNE [L-CC (−) and HNE (+)] (+$P<0.05$ and ++$P<0.01$) are indicated.

obtained 24 h after stimulation with HNE by centrifuging at 400 × *g* for 5 min.

2.2. Measurement of MUC5AC mRNA expression

MUC5AC mRNA expression was measured with real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (Yasuo et al., 2006). Matching primers and hybridization probes were designed by Nihon Gene Research Labs Inc. (Sendai, Japan). Real-time quantitative PCR curves were analyzed by Light Cycler 3.5 software. For the relative quantification of MUC5AC mRNA expressions, the mRNA expression of GAPDH was used as a control.

2.3. Supernatant MUC5AC protein measurement with ELISA

MUC5AC protein concentration in culture supernatants was measured with ELISA methods using a MUC5AC monoclonal antibody (Lab Vision, Fremont, CA) (Yasuo et al., 2006). The data were analyzed using Microplate Manager III, and expressed as the ratio compared with those of the control vehicle (PBS).

2.4. Measurement of reactive oxygen species

To detect the ROS production, intracellular H_2O_2 was measured with Image-iT™ LIVE green reactive oxygen species detection kit (Molecular Probes Inc., Eugene, OR) (Tampo et al., 2003). Green fluorescence from the cells on coverslips (24 mm × 24 mm) in 6-well culture plates was monitored using an Olympus fluorescence microscope (BX-51, Tokyo, Japan, excitation 488 nm, emission 620 nm) and a digital camera system (DP-70, Olympus). The fluorescence intensity was measured and calculated with Adobe Photoshop 5.0 (Adobe systems Inc., San Jose, CA) (Tampo et al., 2003). Because peak production of ROS by HNE was obtained 30 min after stimulation with HNE, we examined the effects of agents on ROS production 30 min after stimulation with HNE.

2.5. Statistical analysis

All data are expressed as means ± standard error (S.E.M.). The data distribution of the variables was assessed with Bartlett's test. Data showing a normal distribution were analyzed with one-way analysis of variance (ANOVA), and data not showing a normal distribution were analyzed with the Kruskal–Wallis test, followed by multiple comparisons among groups with the nonparametric Tukey–Kramer method. Significance was determined at $P<0.05$ (two-tailed test).

3. Results

HNE (100 nM) significantly increased MUC5AC mRNA expression in the cells, and MUC5AC protein concentration in culture supernatants (Fig. 1A and B). L-Carbocisteine alone did not affect levels of MUC5AC mRNA expression and MUC5AC protein concentration in culture supernatants (data not shown). L-Carbocisteine reduced HNE-induced MUC5AC mRNA expression dose-dependently (Fig. 1A). Likewise, L-carbocisteine (10 and 100 μM) significantly reduced MUC5AC protein concentration in culture supernatants induced by HNE stimulation (Fig. 1B).

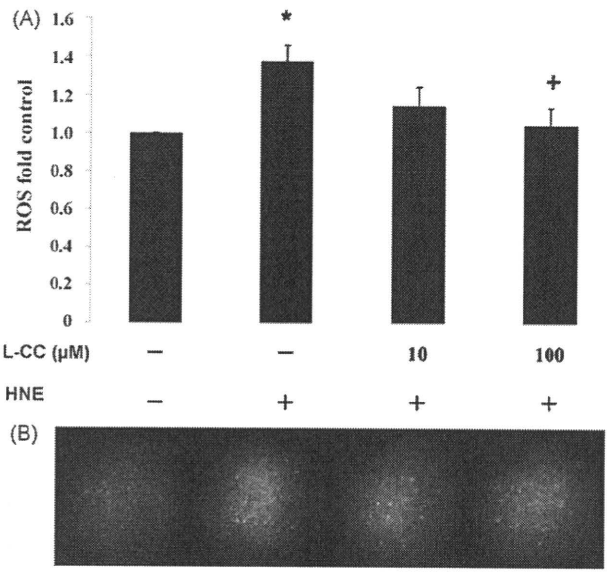

Fig. 2. (A) Intracellular ROS production in NCI-H292 cells 30 min after stimulation with HNE in the presence of L-carbocisteine of vehicle. Results are mean ± S.E.M. from 5 samples. Significant differences from the values exposure to vehicle [control, L-CC (−) and HNE (−)], and from the values exposure to HNE [L-CC (−) and HNE (+)] are indicated by *$P<0.05$ and +$P<0.05$, respectively. (B) Intracellular green fluorescence from ROS produced in NCI-H292 cells 30 min after stimulation with HNE in the presence of L-carbocisteine (L-CC) or vehicle. Data are representative of five different experiments.

The cells produced ROS consistently (Fig. 2A) and ROS production was observed as green staining (Fig. 2B). HNE (100 nM) increased ROS production in the cells (Fig. 2). Furthermore, L-carbocisteine (100 μM) significantly reduced fluorescence intensity induced by HNE (100 nM) which shows ROS production (Fig. 2).

4. Discussion

In this report, we demonstrated that exposure to HNE increased the mRNA and protein expression of MUC5AC in H292 cells. The mucin mRNA expression and production induced by HNE were reduced by incubation with L-carbocisteine. Intracellular ROS production was increased by stimulation with HNE in the cells, and L-carbocisteine reduced the ROS production by HNE.

Fischer and Voynow (2002) demonstrated that HNE induces MUC5AC gene expression via a signaling pathway involving ROS in normal human bronchial epithelial and A549 cells. Furthermore, Shao and Nadel (2005) showed that HNE causes activation of protein kinase C which mediated ROS production, resulting in activation of the TNF-alpha-converting enzyme. Subsequent TGF-α release leads to epidermal growth factor receptor activation and MUC5AC production in normal human bronchial epithelial cells and NCI-H292 cells (Shao and Nadel, 2005). In fact, results in the present study show that HNE increases MUC5AC and produces ROS in NCI-H292 cells, and are consistent with those of these previous reports.

L-Carbocisteine has various functions in the epithelial cells including reduction of pro-inflammatory cytokines after infection of rhinovirus which is the major cause of common colds and COPD exacerbations (Yasuda et al., 2006b). Furthermore, we demonstrated that L-carbocisteine reduced mucin secretion itself which causes airway narrowing and exacerbations in COPD patients. Other than the inhibitory effects on airway inflammation (Yasuda et al., 2006b), a reduced frequency of COPD exacerbations (Yasuda et al., 2006a) and increased QOL in COPD patients treated with L-carbocisteine might also be associated with reduced mucin production by HNE released from neutrophils which accumulate in airways in COPD patients (Macnee, 2007).

In contrast to NAC and ambroxol, L-carbocisteine does not have a SH group which acts as a scavenger of H_2O_2. However, a recent report demonstrated that L-carbocisteine scavenges ROS and inhibits the production of ROS from rat neutrophils (Nogawa et al., 2009), and the effects of L-carbocisteine are similar to those of NAC. These antioxidant effects might relate to the inhibitory effects of L-carbocisteine on HNE-induced mucin production as shown in the present study.

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SURGERY TODAY

Pouchitis Disease Activity Index (PDAI) Does Not Predict Patients with Symptoms of Pouchitis Who Will Respond to Antibiotics

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Abstract

Purpose. To evaluate whether the pouchitis disease activity index (PDAI) alone is sufficient to select appropriate treatment plans for ulcerative colitis patients with bowel movement problems following ileal pouch–anal anastomosis (IPAA).

Methods. The study included 70 patients undergoing an IPAA. For these patients, an evaluation by PDAI was performed prospectively at 1–2 years after the ileostomy closure. When the symptoms relevant to bowel movement appeared, PDAI was evaluated and metronidazole or ciprofloxacin was administered. Pouchitis was diagnosed in patients with PDAI scores of 7 or higher. The patients whose PDAI score was less than 7 and who responded to antibiotic therapy were defined as treatment responders having disease not diagnosed by PDAI (TR-NDPDAI).

Results. Pouchitis was diagnosed in 16 of the 70 enrolled patients (22.9%) using the PDAI scoring system. Of these 16 patients, 11 had acute pouchitis and 5 had chronic pouchitis. Twenty-one patients whose PDAI score was less than 7 were symptomatic. Among them, 12 were TR-NDPDAI. In patients with TR-NDPDAI, antibiotic treatment resulted in significant improvements in the PDAI score ($P < 0.001$) and in clinical symptoms ($P < 0.001$) after treatment.

Conclusion. Antibiotic treatment was effective in a considerable number of ulcerative colitis patients whose PDAI score was less than 7 after IPAA.

Key words Pouchitis · Pouchitis disease activity index · Ulcerative colitis

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Introduction

Since ileal pouch–anal anastomosis (IPAA) was first described,[1] IPAA has become the preferred surgical technique for the treatment of ulcerative colitis (UC). While the surgical advancements for IPAA have reduced the short-term morbidity, long-term complications, such as pouchitis[2], small bowel obstruction,[3] and portal vein thrombi[4] persist as problematic issues.

Pouchitis is an idiopathic inflammatory disease of the ileal reservoir that may occur after IPAA. The frequency of pouchitis varies from 7% to 59%.[4,5] There are no universal diagnostic criteria, and any differences in the diagnostic criteria between institutions may account for the wide range of occurrence. The pouchitis disease activity index (PDAI) was proposed by the Mayo Clinic and has three components as follows: clinical symptoms, endoscopic findings, and pathological findings.[6] A diagnosis of pouchitis made from only the subjective measures of disease activity, such as the symptoms, may lead to an overassessment.[2,6] Therefore, the incorporation of objective data such as endoscopic and pathological findings is considered necessary for an accurate evaluation. The PDAI was therefore found to have the more widely used criteria for the diagnosis of pouchitis and a preferable tool for its clinical study.

However, the diagnostic ability of the PDAI itself is difficult to assess because an absolute diagnosis of pouchitis cannot be made using other diagnostic criteria.[7] In addition, the information from the PDAI does not provide a clear diagnosis and prediction of the best treatment. Pouchitis is often not diagnosed using the PDAI, even in symptomatic patients. Antibiotic therapy is successful in some of these patients not diagnosed by PDAI.[2,8]

The objective of the current study was to examine whether PDAI alone was sufficient to design a treatment strategy for symptomatic patients after IPAA. Two indices, the PDAI and the response to antibiotics,

were used to classify patients who had IPAA and who exhibited bowel movement symptoms.

Patients and Methods

Study Population

The subjects were selected from all patients who had undergone a total colectomy and IPAA for UC with simultaneous temporary ileostomy between January 1997 and December 2003 at the First Department of Surgery, Hiroshima University, and for whom 1–2 years had elapsed since the ileostomy closure. None of these patients had been previously diagnosed with pouchitis by PDAI. Patients were excluded if they were 12 years of age or younger, took antibiotics recently (within 2 weeks before they entered this study), had infection enteritis, or were suspected of having Crohn's disease postoperatively or had any nonfunctioning ileal pouches (e.g., patients with evacuation tubes).

Study Design

Outpatient endoscopy was performed on the patients with or without symptoms 1–2 years after the closure of the ileostomy to evaluate the inflammatory changes of the pouch. A prospective evaluation was performed on the endoscopic findings of ileal pouch mucosa and on the pathological findings from a biopsy. In addition, for patients whose bowel movement symptoms worsened during the study period, an evaluation by PDAI was performed and 750 mg/day metronidazole or 1000 mg/day ciprofloxacin was orally administered for 14 days. The worsening of symptoms was defined as an increase in the PDAI symptoms score of one or greater points. The daily recommended dose of metronidazole is 1200 mg or 15–20 mg/kg.[9] However, we set the dose of metronidazole at 750 mg/day, because UC patients in Japan weigh less than patients in Europe or the United States, and Japanese patients tend to have more gastrointestinal symptoms at the dose used in Europe or the United States. The protocol for this study was conducted with written consent from each participant.

Method of Diagnosis of Pouchitis

The clinical symptoms, endoscopic findings, and pathological findings were assigned scores using the PDAI as proposed by the Mayo Clinic.[6] The PDAI has three components: clinical symptoms, endoscopic findings, and pathological findings. The highest score for each component is 6, and a total score of 7 is considered indicative of pouchitis (PDAI-diagnosed pouchitis). The patients were interviewed and their clinical symptoms were scored during their outpatient visits by the same physician. Endoscopies were performed by one of the three specified endoscopists. The films taken during endoscopy were examined and used to evaluate the endoscopic findings. During the endoscopies, biopsies were taken from the pouches with or without any particular findings of inflammation. The pathological findings were re-examined and scored at the same time in a blinded fashion by one pathologist.

A PDAI endoscopy score of 1 or higher was defined as indicative of positive endoscopic findings, a PDAI pathological score of 3 or higher as indicative of positive pathological findings, and a clinical score of 1 or higher as indicative of positive clinical findings.

Definition of Pouchitis, TR-NDPDAI, Cuffitis, and Irritable Pouch Syndrome

The therapeutic effects were evaluated by examining the changes in clinical symptom during the 2 weeks following the initiation of antibiotic treatment for the symptomatic patients. The outcome was evaluated by PDAI, which is based on the symptomatic, endoscopic, and pathological findings (Table 1). The patients were defined as treatment responders having disease not diagnosed by PDAI if they responded to antibiotics and their PDAI scores were less than 7 (TR-NDPDAI). The responsiveness in TR-NDPDAI was defined as a reduction in the PDAI symptoms score by one or more points within 2 weeks after the antibiotic treatment. Cuffitis was diagnosed in patients with a PDAI score of less than 7 and with significant inflammatory findings in the residual rectum. Irritable pouch syndrome (IPS) was diagnosed in nonresponders with a PDAI score of less than 7 who underwent a mucosectomy or had no inflammatory changes in the residual rectum.[10]

Table 1. List of outcome measurements

Symptomatic findings
Stool frequency
Rectal bleeding
Fecal urgency
Fever
Endoscopic findings
Edema
Granularity
Friability
Loss of vascular pattern
Mucus exudates
Ulceration
Pathological findings
Polymorphonuclear leukocyte infiltration
Ulceration
PDAI score

PDAI, pouchitis disease activity index

Statistical Analysis

A Chi-square test was used for the categorical data (comparisons of patient background data). Student's *t*-test was used for continuous data. The Mann–Whitney *U*-test was used to compare the scores of each PDAI component between the two groups. A comparison between before and after the antibiotic treatment was performed for every component using Wilcoxon's test. Logistic regression models were used to determine which components were significantly related to PDAI scores less than 7 in TR-NDPDAI in comparison to pouchitis. A P value of 0.05 or less was considered to be statistically significant. All data are indicated as the mean ± standard deviation.

Results

Within the study period, 102 patients underwent surgeries for UC. Each surgery was performed by the same surgeons. A hand-sewn IPAA with a mucosectomy was performed in 65 patients and a double-stapled technique was used in 5 patients. In 20 patients and 12 patients, we were unable to obtain consent to perform postsurgical endoscopy and biopsies during endoscopy, respectively. All of these patients were asymptomatic. Therefore, a total of 32 patients were excluded, and the remaining 70 patients were entered into the study. The median follow-up period at the endoscopic examination in these 70 patients was 22 months (range, 12–81 months) after the ileostomy closure.

Among the 70 patients, 37 (52.9%) were symptomatic, all of whom were treated with antibiotics. The treatment was successful in 23 of these 37 patients. Of the 70 patients, 16 (22.9%) had PDAI-diagnosed pouchitis (PDAI scores of 7 or higher); however, the occurrence rate was 15.7% of the 102 eligible patients, including the asymptomatic patients who did not consent to the endoscopic examinations and biopsies. There were 12 TR-NDPDAI (8 with positive endoscopic findings [>1 point] and 4 with positive pathological findings [>3 points]). Six of the 12 patients were unaware of the worsening of their symptoms because the deterioration was gradual. We found that their symptoms were worsening only after checking previous data in their medical records. Of the 12 TR-NDPDAI, 8 (67%) had objective evidence based on endoscopy and 4 (33%) had objective evidence based on the pathological findings.

Table 2 shows the demographic characteristics of all study patients. There were no significant differences between the three groups with respect to age, gender, the duration of disease before surgery, dose of steroid, or surgical indication. Figure 1 shows the diagnostic tree for each patient group. Of the 16 PDAI-diagnosed pouchitis patients, 11 had acute pouchitis and 5 had chronic pouchitis. Of the 37 symptomatic patients, 21 had PDAI scores of less than 7; 12 of these 21 patients were TR-NDPDAI and 9 were nonresponders. Furthermore, 2 of these 9 patients had cuffitis and the other 7 patients had IPS. During the study period, 33 patients were asymptomatic and therefore were not suspected of having pouchitis that required treatment. The PDAI score was 2.8 ± 1.1 for the asymptomatic patients and 6.9 ± 2.8 for the symptomatic patients ($P < 0.001$).

Figure 2 shows the changes in the pretreatment and post-treatment scores of 12 TR-NDPDAI patients. The PDAI score and the clinical symptom score showed a significant improvement (total PDAI score, 5.0 ± 1.1 vs 3.2 ± 1.1 [$P = 0.002$]; clinical symptoms score, 1.6 ± 0.8

Table 2. Demographic characteristics of ulcerative colitis patients after IPAA

	Pouchitis ($n = 16$)	TR-NDPDAI ($n = 12$)	Nonpouchitis ($n = 42$)	P value
Median age (range)	38 (23–64)	39 (24–68)	33 (19–62)	>0.212*
Male:female ratio	9:7	4:8	18:24	0.462
Extent of colitis (pancolitis:left side involved colitis)	13:3	12:0	32:10	0.174
Median total doses of preoperative steroid, g (range)	10.0 (2–26.6)	15.0 (12.9–32.4)	10.8 (1–64.8)	>0.068*
Median duration of UC before operation, months (range)	55 (10–324)	72 (21–156)	84 (3–128)	>0.259*
Indication of surgery				
toxic colitis or perforation or massive bleeding	4	3	13	0.872
cancer	1	0	0	
refractory to medical treatment	11	9	29	
Operation (2-stage:3-stage)	5:11	6:6	13:29	0.452
Mucosectomy:double stapler method	16:0	11:1	38:4	0.446
Median follow-up months at endoscopy (range)	15 (12–80)	24 (12–80)	24 (12–81)	>0.408*
Metronidazole:Ciprofloxacin	12:4	10:2	9:0	

IPAA, ileal pouch–anal anastomosis; PDAI, pouchitis disease activity index; TR-NDPDAI, treatment responders having disease not diagnosed by PDAI

P value represents the results of Chi-square test unless otherwise specified

* Student's *t*-test

vs 0.2 ± 0.4 [P = 0.002]). Since the scores for the endoscopic findings were initially low in TR-NDPDAI, there was no significant improvement observed after the treatment (1.0 ± 1.0 vs 0.8 ± 1.1; P = 0.157). In addition, the scores for the pathological findings did not reveal any improvement (2.4 ± 0.7 vs 2.2 ± 0.4; P = 0.083).

The mean total PDAI score and each component score in the patients with PDAI-diagnosed pouchitis and in TR-NDPDAI are compared in Table 3. There were no significant differences between the two groups with respect to their clinical symptoms score (2.7 ± 1.4 vs 1.9 ± 0.8; P = 0.103). However, TR-NDPDAI had significantly lower scores for the endoscopic and pathological findings (endoscopic findings: 3.3 ± 1.7 vs 1.0 ± 1.0 [P = 0.001]; pathological findings: 3.5 ± 0.9 vs 2.4 ± 0.7 [P = 0.003]). The contributor to the low PDAI score in TR-NDPDAI was the particularly low score for the endoscopic and pathological findings.

In the univariate logistic regression models, the scores for the endoscopic and pathological findings were the main predictors of a PDAI score 7 or higher in PDAI diagnosed pouchitis in comparison to TR-NDPDAI (endoscopic findings: odds ratio 3.257, 95% confidence interval [1.319–8.044]; pathological findings: odds ratio

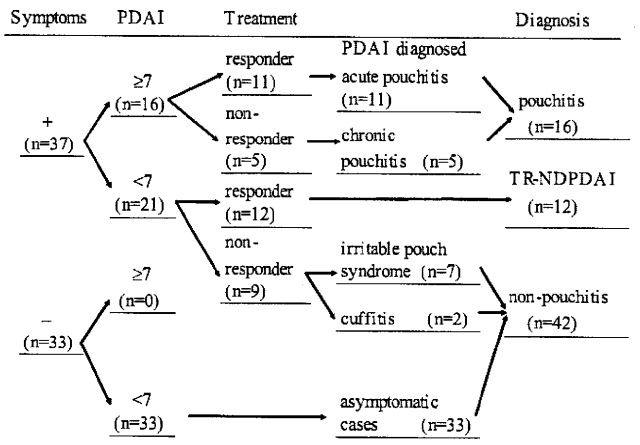

Fig. 1. Patient classification by pouchitis disease activity index (*PDAI*) score and response to antibiotic treatment. *TR-NDPDAI*, treatment responders having disease not diagnosed by PDAI

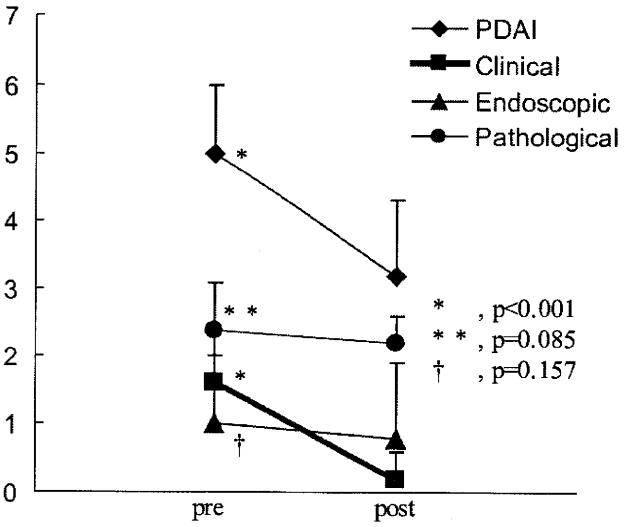

Fig. 2. Changes in PDAI scores and each component score of PDAI in TR-NDPDAI between pre- and post-treatment

Table 3. Comparison of total PDAI score and each component score in patients with pouchitis and TR-NDPDAI

	PDAI-diagnosed pouchitis (n = 16)	TR-NDPDAI (n = 12)	P value
PDAI	9.4 ± 2.4	5.3 ± 0.7	<0.001
Clinical	2.7 ± 1.4	1.9 ± 0.8	0.103
stool frequency	1.4 ± 0.6	1.3 ± 0.5	0.525
bleeding	0.5 ± 0.5	0.2 ± 0.4	0.074
urgency	0.4 ± 0.5	0.3 ± 0.5	0.491
fever	0.4 ± 0.5	0.2 ± 0.4	0.236
Endoscopic inflammation	3.3 ± 1.7	1.0 ± 1.0	0.001
edema	0.8 ± 0.4	0.2 ± 0.4	0.003
granularity	0.3 ± 0.5	0.0 ± 0.0	0.036
friability	0.6 ± 0.5	0.0 ± 0.0	0.002
loss of vascular pattern	0.8 ± 0.4	0.2 ± 0.4	0.003
mucous exudate	0.2 ± 0.4	0.0 ± 0.0	0.120
ulcer	0.7 ± 0.5	0.7 ± 0.5	0.909
Pathological inflammation	3.5 ± 0.9	2.4 ± 0.7	0.003
polymorphonuclear leukocyte inflammation	1.7 ± 0.5	1.2 ± 0.4	0.007
ulceration per low-power field	1.8 ± 0.7	1.3 ± 0.5	0.019

Values reported are mean ± one standard deviation
PDAI, pouchitis disease activity index; TR-NDPDAI, treatment responders having disease not diagnosed by PDAI
P value represents the results of the Mann–Whitney U-test

Table 4. Comparison of total PDAI score and each component scores between each subgroups

	PDAI ≥ 7			PDAI < 7		
	Acute pouchitis (n = 11)	Chronic pouchitis (n = 5)	TR-NDPDAI (n = 12)	IPS (n = 7)	Cuffitis (n = 2)	Asymptomatic cases (n = 33)
PDAI	9.0 ± 2.2*	10.4 ± 2.7	5.3 ± 0.7	5.2 ± 1.1	4.5 ± 0.7	2.8 ± 1.1†
Clinical symptoms	2.9 ± 1.2*	2.2 ± 1.9	1.9 ± 0.8	1.1 ± 0.4**	2.5 ± 0.7	0.0 ± 0.0†
Endoscopic findings	2.6 ± 1.6*	4.6 ± 1.1	1.0 ± 1.0	1.3 ± 1.3	0.0 ± 0.0	10.3 ± 0.6††
Pathological findings	3.5 ± 1.0*	3.6 ± 0.5	2.4 ± 1.7	2.8 ± 0.9	2.0 ± 0.0	2.5 ± 0.7

Values reported are mean ± one standard deviation
PDAI, pouchitis disease activity index; TR-NDPDAI, treatment responders having disease not diagnosed by PDAI; IPS, irritable pouch syndrome
P value represents the results of the Mann–Whitney U-test. * $P < 0.001$ (vs TR-NDPDAI); ** $P = 0.028$ (vs TR-NDPDAI); † $P < 0.001$ (vs TR-NDPDAI); †† $P = 0.015$ (vs TR-NDPDAI)

4.645,95% confidence interval [1.520–14.200]). However, the clinical symptoms score was not a significant factor (symptoms: odds ratio 1.761, 95% confidence interval [0.869–3.571]).

Table 4 shows the scores of the subgroups that were classified on the basis of the PDAI score and response to antibiotics. There was no significant difference in the PDAI score between the TR-NDPDAI and IPS groups (5.3 ± 0.7 vs 5.2 ± 1.1; $P = 0.857$). There was a significant difference only in the clinical symptoms score (1.9 ± 0.8 vs 1.1 ± 0.4; $P = 0.028$), but not in the objective scores for the endoscopic findings (1.0 ± 1.0 vs 1.3 ± 1.3; $P = 0.505$) or pathological findings (2.4 ± 1.7 vs 2.8 ± 0.9; $P = 0.253$).

There was also no significant difference in the pathological findings between the TR-NDPDAI and asymptomatic patients (2.4 ± 1.7 vs 2.5 ± 0.7; $P = 0.890$). The clinical symptoms and endoscopic findings scores were significantly higher in TR-NDPDAI than in the asymptomatic patients (symptoms: 1.9 ± 0.8 vs 0.0 ± 0.0 [$P < 0.001$]; endoscopic findings: 1.0 ± 1.0 vs 0.3 ± 0.6 [$P = 0.015$]). In the two cases of cuffitis, the endoscopic findings score was 0.

Discussion

Various problems have been identified in the PDAI,[6–9,11–13] and some have proposed that the objective findings such as endoscopy and pathology should be emphasized more than the subjective assessment of clinical symptoms.[9,11] On the other hand, in one report the clinical symptoms of chronic pouchitis improved following treatment with metronidazole, while the objective (endoscopy and pathology) findings did not,[13] thus suggesting that the therapeutic effect cannot effectively be determined from the objective findings. Furthermore, various problems have been identified in relating the assessment by the pathological findings to the PDAI.[7,12] Shen et al. stated that the sensitivity and specificity of the modified PDAI, which excludes the pathological findings, should be accepted[7] and that biopsies are probably unnecessary from the viewpoint of economic efficiency.[13] From the Japanese group, the Japanese criteria, which consisted of the clinical symptoms and endoscopic findings and did not contain any pathological findings and scoring system, have been proposed as more simple and useful criteria in clinical practice.[14] In this study, we selected the PDAI as having the most widely used criteria to evaluate the pathological findings and severity.

Furthermore, Heuschen et al.[9] suggested that the cutoff of 7 points is too high for the diagnosis of pouchitis and that if the cutoff point were one or two points lower, the PDAI would be more accurate. In addition, a question has been raised with respect to the treatment of the patients with PDAI scores of less than 7 but who are responsive to antibiotics.[8] Therefore, in the current study we combined two indices (namely, the diagnosis using the PDAI and the estimation according to the response to antibiotics) and then subclassified the symptomatic patients. We thus found a significant number of cases in which antibiotic treatment was effective in patients with low PDAI scores. Of the 70 patients, 37 were symptomatic but only 16 had PDAI-diagnosed pouchitis. Of the remaining 21 symptomatic patients, 12 of 21 (57.1%) responded to antibiotics. Therefore, using the PDAI score (>7), 12 patients who were relieved of their symptoms by antibiotics were overlooked. Although the PDAI is a useful way of objectively quantifying the severity of pouchitis, it did not predict the success of treatment in our study.

We considered that TR-NDPDAI included symptomatic patients who were not diagnosed by the PDAI scoring system and the patients with proximal small bowel bacterial overgrowth. Multiple studies have shown that some patients with irritable bowel syndrome, analogous to IPS, have small bowel bacterial overgrowth, and this is thought to occur in 40% of the patients with ileal pouches.[15]

We examined where the differences in the PDAI scores lie between the TR-NDPDAI and the PDAI-diagnosed pouchitis group. Although no significant difference was observed in the clinical symptoms, a large difference in the endoscopic and pathological findings contributed to the difference in the total PDAI scores among these two groups. PDAI-diagnosed pouchitis occurred in 22.9% of all patients in this study and 15.7% of all eligible patients including those excluded because they did not give consent for endoscopy due to an absence of symptoms. Since some TR-NDPDAI may have pouchitis, the incidence of pouchitis may be higher than this value.

Shen et al. defined IPS by the presence of low PDAI scores in symptomatic patients and stated that the treatment for irritable bowel syndrome was successful in these patients, and reported that a very high frequency (42.6%) of symptomatic patients had IPS.[11] The frequency is high because IPS is a diagnosis that covers a wide range of nonspecific inflammatory conditions with symptoms similar to pouchitis.[16] Instead, we classified these symptomatic patients with a PDAI score of less than 7. The patients who were successfully treated with antibiotics were classified as TR-NDPDAI (12 cases). Cuffitis was diagnosed in patients not successfully treated with antibiotics who had inflammation in the residual rectum and had normal endoscopic findings in the pouch (2 cases). Irritable pouch syndrome was diagnosed in all the other patients (7 cases). Although we examined the differences between the TR-NDPDAI and nonpouchitis groups, in the objective findings there was little difference between the TR-NDPDAI and nonpouchitis groups, as shown in Table 4.

From the perspective of treatment, TR-NDPDAI patients must be differentiated from IPS patients. Therefore, in regard to the etiology we thought that the IPS was analogous to irritable bowel syndrome as Shen et al. described,[11] and TR-NDPDAI was analogous to pouchitis. Although TR-NDPDAI had slightly higher clinical symptom scores than IPS patients, the differences in the scores of total PDAI, endoscopic findings, or pathological findings were not significant. Therefore, the differentiation between the two groups is difficult using the components of the PDAI. It may be a treatment option to administer anticholinergic or antidepressant agents to the symptomatic patients with PDAI scores less than 7 before the administration of antibiotics. However, it is not clear whether the treatment strategy for IPS would be less costly with fewer side effects than the use of antibiotics. At this time, we cannot recommend which treatment strategy should be initiated first.

The benefits of a mucosectomy are the lower rates of inflammation and dysplasia in the retained mucosa in UC. However, a recent meta-analysis suggested that nighttime seepage of stool, and resting and squeeze pressure were worse after a mucosectomy than stapled anastomosis.[17] A high percentage of patients undergoing a mucosectomy and hand-sewn anastomosis in this study may contribute to the high incidence of symptomatic patients other than PDAI-diagnosed pouchitis.

This study is limited by the absence of a placebo treatment arm to understand how much the extent of the improvement was due to antibiotics versus a mild random variation in the symptoms, especially in TR-NDPDAI. In conclusion, the PDAI was not a good indicator of the success of treatment in symptomatic patients who have undergone IPAA. A considerable number of symptomatic patients with PDAI scores less than 7 showed clinical improvement upon antibiotic treatment. Because the symptoms after IPAA had several causes, an investigation of appropriate treatment for each of the conditions is necessary. For this reason, we believe that a detailed analysis should be performed to develop treatment plans in symptomatic patients even if their PDAI scores are less than 7.

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Short Communication

H5N1-Infected Cells in Lung with Diffuse Alveolar Damage in Exudative Phase from a Fatal Case in Vietnam

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SUMMARY: Necropsied lung tissues of three fatal cases with avian influenza A virus (H5N1) infection in Vietnam were analyzed to detect H5N1 virus-infected cells. Formalin-fixed and paraffin-embedded lung tissue sections showed typical histological features of diffuse alveolar damage (DAD) in all cases. Immunohistochemistry for the influenza A virus nucleoprotein antigen revealed positive signals of bronchiolar and alveolar epithelial cells in only one patient, who exhibited DAD with an exudative phase and died on the 6th day after onset. However, no signal was detected in the other two cases of DAD with a proliferative phase. These patients died on day 16 and day 17 after onset, respectively. H5N1 virus antigens were detected predominantly in epithelial cells in terminal bronchioles and in alveoli, i.e., type I and type II alveolar pneumocytes, and in alveolar macrophages. The pathogenesis of exudative DAD caused by H5N1 infection is discussed.

Highly pathogenic avian influenza A H5N1 virus (H5N1) infection has been reported to cause severe respiratory disease. In 1997, H5N1 was first isolated in Hong Kong from tracheal aspirates of a 3-year-old boy with a fatal respiratory illness (1-3). In 2003, human disease associated with H5N1 re-emerged (4). Since then, the number of confirmed fatal human H5N1-infected cases has increased and now totals approximately 200 cases. These cases occurred, predominantly, in Vietnam, Thailand, and Indonesia (5-9). The histopathological data for H5N1 virus infection in humans were, however, limited (3,4,6,8,10-12), and the pathogenesis of the disease remains unclear. Examination of ex vivo infected lung tissues showed that influenza A virus nucleoprotein (InfA-NP) was detected in pneumocytes and in alveolar macrophages (13). Also the pattern of viral attachment in human respiratory tract sections showed that H5N1 attached to the apical cell membrane of bronchiolar cells, type II pneumocytes and alveolar macrophages (14,15). The postmortem study of H5N1-infected patients has recently been published for the first time (16).

In the present study, we describe the histopathological findings from three fatal cases of H5N1 infection from the National Hospital of Pediatrics in Hanoi, Vietnam. The detailed clinical findings of Case 1 and Case 2 have been described previously (5). On admission, all patients presented with fever, cough, and dyspnea, and H5N1 virus was detected in tracheal fluids by reverse-transcriptase polymerase chain reaction (RT-PCR) before death occurred. The duration of the disease in Case 1, 6 days, was much shorter than in the other two cases (Table 1). Small pieces of lung tissues in the lower respiratory tract were necropsied and histological and immunohistochemical examinations were carried out on formalin-fixed and paraffin-embedded lung tissues.

The hematoxylin and eosin-stained lung sections of Case 1 demonstrated typical histological features of diffuse alveolar damage (DAD) with an exudative phase (Fig. 1a). Eosinophilic hyaline membrane was found on alveolar ducts and on alveoli. The alveolar space was filled with proteinaceous exudates containing erythrocytes, macrophages, and cell debris. The alveolar septa were thickened by edema with mild inflammatory infiltration, consisting of lymphocytes and macrophages. In Cases 2 and 3, hyaline membrane formation was focally found, and the proliferation of fibroblasts in the interstitial space was marked in comparison to Case 1. Mild interstitial inflammation and proliferation of type II pneumocytes with bizarre and cuboidal features were observed (Fig. 1c), indicating that Cases 2 and 3 were in the proliferative (repair) phase of DAD. Squamous cell metaplasia in the bronchiolar epithelium was also observed (Fig. 1d). Focal accumulation of neutrophils in the alveolar space was found in Case 3, suggesting pulmonary bacterial infection. These histological features were similar to those reported previously in fatal human H5N1 influenza A virus-infected cases (4,8,10,11).

To detect the influenza A virus antigen, the sections were immunostained with an avidin-biotin complex immunoperoxidase method (LSAB2 kit/HRP/DAB; Dako Cytomation, Copenhagen, Denmark) using a mouse monoclonal antibody against InfA-NP (17). Positive signals for InfA-NP were detected in 6 of 6 blocks of lung tissue from Case 1, whereas they were not found in those from Case 2 or 3. The signals were found mainly in alveolar epithelial cells and in interstitial cells (Fig. 1b). The many positive cells were interpreted as type II pneumocytes and/or alveolar macrophages, but the positive cell presented in the inset in Fig. 1b was considered to be a type I pneumocyte based on its histological location and morphology. H5N1-RNA was also detected by real-time RT-

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**Deceased after the contribution of this study.

Table 1. Histopathological findings in the lung of H5N1 fatal cases in Vietnam

Case	Age (y)/ Sex	Days from onset to death	Histology in lung sections	RT-PCR for H5N1 (tracheal fluids)	RT-PCR for H5N1 (paraffin-embedded sections of lung)	Immunohistochemistry for InfA-NP antigen and co-localization with cell marker proteins
1[1)]	12/F	6	DAD with an exudative phase, Hyaline membrane formation Hemmorhagic necrosis	Positive	Positive	Positive for InfA-NP antigen, and colocalized with AE1/AE3, EMA, SPA, SPD, CD68, CD34
2[2)]	5/M	17	DAD with a proliferative (repair) phase Hyaline membrane formation	Positive	Negative	Negative for InfA-NP antigen
3	4/M	16	DAD with a proliferative (repair) phase Hyaline membrane formation Microabscess	Positive	Negative	Negative for InfA-NP antigen

[1)]: Patient 1 in Ref (5).
[2)]: Patient 2 in Ref (5).
M, male; F, female; DAD, diffuse alveolar damage; InfA-NP, influenza virus A nucleoprotein; EMA, epithelial membrane antigen; SPA, surfactant protein A; SPD, surfactant protein D.

Table 2. Antibodies used for double immunofluorescence staining

Antigen	Antibody type	Stained cells	Source
cytokeratin (AE1/AE3)	mouse monoclonal	epithelial cell of bronchiole	Dako
epithelial membrane antigen (EMA)	mouse monoclonal	epithelial cell	Dako
surfactant apoprotein A (SPA)	mouse monoclonal	type II alveolar pneumocyte	Dako
surfactant apoprotein D (SPD)	rabbit polyclonal	type II alveolar pneumocyte	Chemicon[1)]
CD68 (KP1)	mouse monoclonal	alveolar macrophage	Dako
CD68 (PG-M1)	mouse monoclonal	alveolar macrophage	Dako
CD34	mouse monoclonal	endothelial cell	Immunotech[2)]
influenza A virus nucleoprotein	mouse monoclonal	influenza A virus infected cell	in-house Ref. (17)
influenza A virus nucleoprotein	rabbit polyclonal	influenza A virus infected cell	in-house Ref. (17)

[1)]: Chemicon, Temecula, Calif., USA.
[2)]: Immunotech, Marseille, France.

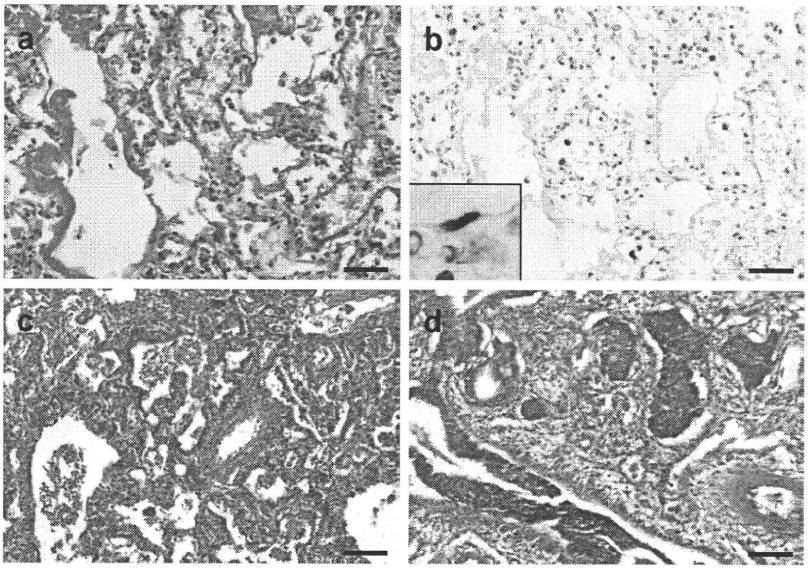

Fig. 1. Hematoxylin and eosin stainings and immunohistochemistry for influenza virus A nucleoprotein (InfA-NP) in Case 1. (a) Hyaline membrane formation is observed on the alveolar walls. In the interstitial space, edema and mild inflammatory cell infiltrates are observed (Case 1). (b) InfA-NP antigens are detected in alveolar epithelial cells and in the interstitial space. InfA-NP-positive, type I pneumocyte is indicated in the inset. (c) Mild interstitial inflammation and proliferation of type II pneumocytes with bizarre and cuboidal features were observed (Case 3). (d) Squamous cell metaplasia in the bronchiolar epithelium was also observed (Case 2). Scale bar = 100 μm.

PCR in paraffin-embedded lung sections from Case 1 only (18). In DAD with a proliferative phase, as in Cases 2 and 3, viral antigens and nucleic acids were not detected.

To characterize virus-infected cells, confocal laser scanning microscopy was used to visualize double immunofluorescence staining for InfA-NP and for cell-type specific marker proteins of epithelial cells, macrophages, and endothelial cells. The antibodies used are shown in Table 2. Alexa Fluor 568-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes, Eugene, Oreg., USA) and Alexa Fluor 488-conjugated anti-rabbit or anti-mouse IgG (Molecular Probes) were used as secondary antibodies. InfA-NP signals were detected most

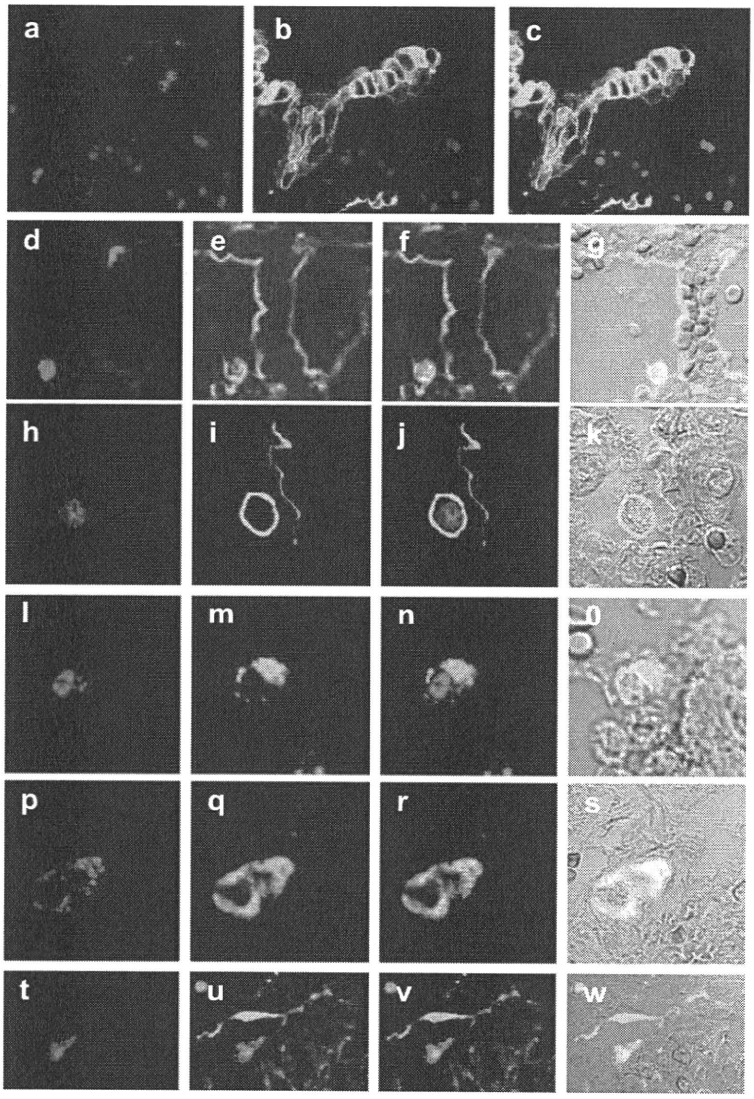

Fig. 2. The phenotype of influenza virus A nucleoprotein (InfA-NP) positive cells. InfA-NP immunoreactivity (a, d, h, l, p, t) (red color) and cytokeratin (b), EMA (e), SPD (i), CD68 (Kp1) (m), CD68 (PGM-1) (q) or CD34 (u) immunoreactivity (green color). Co-localization is presented respectively (c, f, j, n, r, v). Differential interference contrast (DIC) images are also shown (g, k, o, s, w). Original magnifications, ×400.

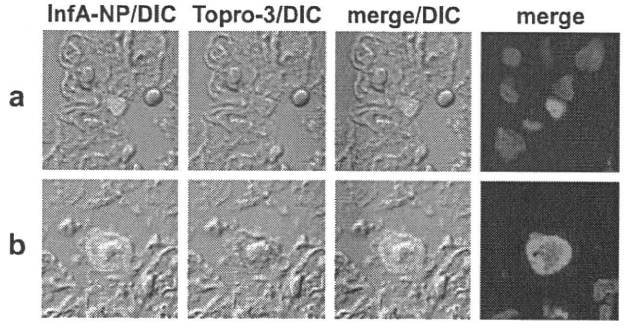

Fig. 3. Immunofluorescence staining of InfA-NP antigen in infected epithelial cells. InfA-NP immunoreactivity (red color), TO-PRO-3 nucleic acid staining (blue color) and merged images (pink color) are shown. Some were analyzed with differential interference contrast (DIC) images. The InfA-NP antigen was localized in nuclei (a) or in cytoplasm (b). Original magnifications, ×400.

frequently in epithelial (EMA-positive) cells. They were also detected in AE1/AE3, SPD, SPA, and CD68-positive cells (Fig. 2), indicating that H5N1 virus antigens were present predominantly in the epithelial cells in terminal bronchioles and alveoli, mainly in type II alveolar pneumocytes and in alveolar macrophages. A few H5N1 virus-infected type I pneumocytes were also suggested by double-positive staining for InfA-NP and for EMA, in combination with distinctive morphology. Although the number was very few, the InfA-NP signal was also detected in CD34-positive cells, suggesting that the H5N1 had infected some CD34-positive endothelial cells. Further investigation will be necessary to confirm the H5N1 infection of human endothelial cells, as has been observed in the endothelial cells of chickens and other birds (19). The localization of InfA-NP antigen within the cell was determined by counterstaining with TO-PRO-3 nucleic acid staining (Molecular Probes). Some InfA-NP signals were detected in nuclei (Fig. 3a) and others were detected in the cytoplasm (Fig. 3b). Histologically, in the early phase of infection, InfA-NP antigen was localized in the nucleus, while in the late phase of infection, InfA-NP antigen was localized in the cytoplasm (20). These observations suggested that viruses were in the proliferative stage in the early phase of H5N1 infection. The histopathological data

are summarized in Table 1.

Avian influenza viruses have been found to preferentially bind to sialic acid-α-2,3-Gal (SAα2-3)-linked oligosaccharides, while human influenza viruses were found to bind to SAα2-6-linked oligosaccharides (21), although these findings were made in vitro or ex vivo experiments. As an in vivo examination, we performed an analysis with the double-staining technique using a monoclonal antibody against InfA-NP in combination with either biotinylated *Maackia amurensis* agglutinin (MAA) lectin (Vector Laboratories, Burlingame, Calif., USA) which is specific for SAα2-3-linked oligosaccharides, or with *Sambucus nigra* agglutinin (SNA) lectin (EY Laboratories, San Mateo, Calif., USA), which is specific for SAα2-6-linked oligosaccharides. In the alveoli, many cells were not stained by SNA lectin but were stained by MAA lectin, suggesting that they express SA α 2-3-linked oligosaccharides, as found in previous reports (21). Unexpectedly, the InfA-NP-positive cells were not double-stained by MAA lectin.

Although the materials were restricted to small pieces of lung tissue in the lower respiratory tract, the evidence in the present study showed that several types of cells in the lung, namely type I and type II alveolar pneumocytes, epithelial cells in terminal bronchioles, macrophages in the alveolar space and CD34-positive endothelial cells in the interstitial tissues, were involved in the disease. The evidence in Case 1, the case with H5N1 infection who died on day 6 after onset, strongly suggests that H5N1 may infect the epithelial cells of alveolar tissues in the early clinical phase and can thereafter be transmitted to adjacent cells. The dissemination of infection among these cells was supposed to be accompanied by the release of pro-inflammatory cytokines from the infected alveolar macrophages (4,10,12), resulting in rapid progression from DAD with an exudative phase to that with a proliferative phase.

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Short communication

Ribavirin inhibits Borna disease virus proliferation and fatal neurological diseases in neonatally infected gerbils

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ABSTRACT

By using neonatal gerbils, we assessed the effect of ribavirin on the proliferation of Borna disease virus (BDV) in the brain. The intracranial inoculation of ribavirin reduced viral propagation in the acutely infected brain, resulting in protection from fatal neurological disorders. We found that the treatment with ribavirin markedly reduces the numbers of OX-42-positive microglial cells, but does not activate expression of Th1 cytokines, in BDV-infected gerbil brains. Our results suggested that ribavirin directly inhibits BDV replication and might be a potential tool for the treatment of BDV infection.

Borna disease virus (BDV) induces Borna disease (BD) in naturally infected horses and sheep, which is characterized by severe non-purulent meningoencephalitis with massive perivascular and parenchymal infiltration (Ikuta et al., 2002a,b; Rott and Becht, 1995). Numerous reports have demonstrated that asymptomatic natural infections of BDV occur worldwide in a variety of vertebrate species, suggesting that the host range of this virus includes all warm-blooded animals (Ludwig and Bode, 2000; Tomonaga et al., 2002). In addition, mounting evidence suggests that humans could be a target for BDV infection (Billich et al., 2002; Carbone, 2001; Ikuta et al., 2002a,b), indicating that BDV presents a possible risk as a zoonotic pathogen. At present, several drugs have been reported to have antiviral effects in BDV infection (Bajramovic et al., 2002, 2004; Bode et al., 1997; Volmer et al., 2005). Bajramovic et al. (2002, 2004) demonstrated that a nucleoside analog, 2′-fluoro-2′-deoxycytidine, as well as 1-β-D-arabinofuranosylcytosine, inhibited the replication and spread of BDV in cell culture and in vivo systems. Some reports have showed that amantadine is effective against a strain of BDV both in vitro and in vivo, although the effect of this drug on anti-BDV activity is still controversial (Bode et al., 1997; Hallensleben et al., 1997; Stitz et al., 1998).

Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, is a synthetic ribonucleoside analog that displays broad-spectrum antiviral activity and is currently used for the treatment of a wide range of DNA and RNA virus infections (Cameron and Castro, 2001; Parker, 2005). Previous studies revealed that ribavirin has an antiviral effect on BDV infection in neural and non-neural cell lines (Jordan et al., 1999; Mizutani et al., 1998). Treatment with ribavirin drastically reduced the levels of viral transcription and release in persistently infected cultured cells, suggesting this agent to be a good candidate for an anti-BDV drug. The effect of ribavirin against BDV-induced neurological disorders has also been examined in a rat model of persistent BDV infection (Solbrig et al., 2002). Adult Lewis rats were infected with BDV and received a daily intraventricular ribavirin injection from 21 days postinfection (p.i.), at which time the infected animals had developed BD-like symptoms. Interestingly, the intraventricular injection of ribavirin caused clinical improvement without changing the viral titer or RNA level in the rat brain. In addition, decreased numbers of microglia, as well as $CD4^+$ and $CD8^+$ T-cells, were observed in the brains of ribavirin-treated, persistently infected rats (Solbrig et al., 2002). From this observation, it has been concluded that ribavirin may reduce the morbidity of BD by impacting on microglial proliferation and its effects on the brain. However, the direct effects of ribavirin on the

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proliferation of BDV in the brain had not been made clear because the viral titer was unchanged in the rat brain by the ribavirin. In the present study, therefore, we assessed the effect of ribavirin on the proliferation of BDV in the brains by using acutely BDV-infected gerbil model.

Gerbils make an intriguing model for analyzing the neuropathogenesis of BDV (Lee et al., 2003; Watanabe et al., 2001, 2003). In previous studies, we demonstrated that despite the development of fatal neurological disorders and aggressive proliferation of BDV in newborn gerbil brains, no severe neuroanatomical alterations were observed (Watanabe et al., 2001, 2003). Furthermore, treatment with an immunosuppressant, cyclosporine A, did not inhibit the fatal disorders in BDV-infected neonatal gerbils (Watanabe et al., 2003). These results indicated that significant replication of BDV in specific areas of the central nervous system (CNS), but not the host immune response, contributes to the onset of the neurological diseases in newborn gerbils. From these observations, we concluded that the gerbil provides a unique model for understanding the direct damage to the CNS caused by the replication of BDV. Thus, the acutely infected neonatal gerbil is a good system with which to evaluate the effects of ribavirin on the proliferation of BDV in the brain.

To determine the dose of ribavirin to inject into neonatal gerbils (SLC, Shizuoka, Japan), we intracranially inoculated ribavirin in phosphate-buffered saline (PBS) into the subarachnoid cavity, which is the interval between the arachnoid membrane and pia mater, at the temporal position by using a microsyringe having two-step needle, which has a 2 mm × 0.4 mm diameter long piercing tip from postnatal day (PD) 10 at 3-day intervals. We used doses of 1.0, 5.0 or 10 mg/(kg shot) in a volume of 10 μl for 15 days. All animal experiments conformed to the guide for the care and use of laboratory animals of the Research Institute for Microbial Diseases, Osaka University. The newborn gerbils endured even the 10 mg/(kg shot) treatment without any weight loss or death (data not shown). Thus, the 10 mg/(kg shot) treatment was adopted in all following experiments. This dose is quite similar to the clinical dosages used in human cases (Carlsson et al., 2008; Engler et al., 2004; Tomoda et al., 2003). Since brain development and/or BDV expansion within the brain could affect the effectiveness of ribavirin, we next compared the starting day of ribavirin administration in acutely BDV-infected newborn gerbils. Newborn gerbils were intracerebrally inoculated at the left temporal position with 4 μl of 200 focus forming units (FFU) of BDV strain He/80 per animal within 24 h after birth (Watanabe et al., 2001) and then injected ribavirin (10 mg/(kg shot)) from either PD10, PD13, or PD16 at 3-day intervals as described above. The animals were monitored for changes in weight and clinical signs of neurological disorders and sacrificed at 25 days p.i., because previous studies revealed that a neonatal infection of 200 FFU BDV induces severe neurological disorders by PD25 (Watanabe et al., 2001, 2003). The gerbils injected with ribavirin from PD10 (P10-Rv) did not lose body weight following the infection, while the infected gerbils treated with ribavirin from PD13 (P13-Rv) and PD16 (P16-Rv) did lose weight, similar to the untreated, infected animals, BDV/Rv(−), from 16 days p.i. (Fig. 1A). A humoral immune response to BDV was detected in all the infected gerbils at PD25 (Table 1). In addition, only 1 of 8 animals (12.5%) had developed signs of neurological disease in P10-Rv gerbils by 25 days p.i., despite that all of the untreated animals developed fatal disorders (Table 1). On the other hand, 33.3 and 50% of the P13-Rv and P16-Rv gerbils, respectively, showed signs of neurological disease (Table 1).

We next investigated whether viral proliferation is inhibited by the administration of ribavirin in the brains of acutely BDV-infected gerbils. The P10-Rv gerbils were sacrificed at PD25 and the BDV RNA level in the cerebral cortex was determined by semi-

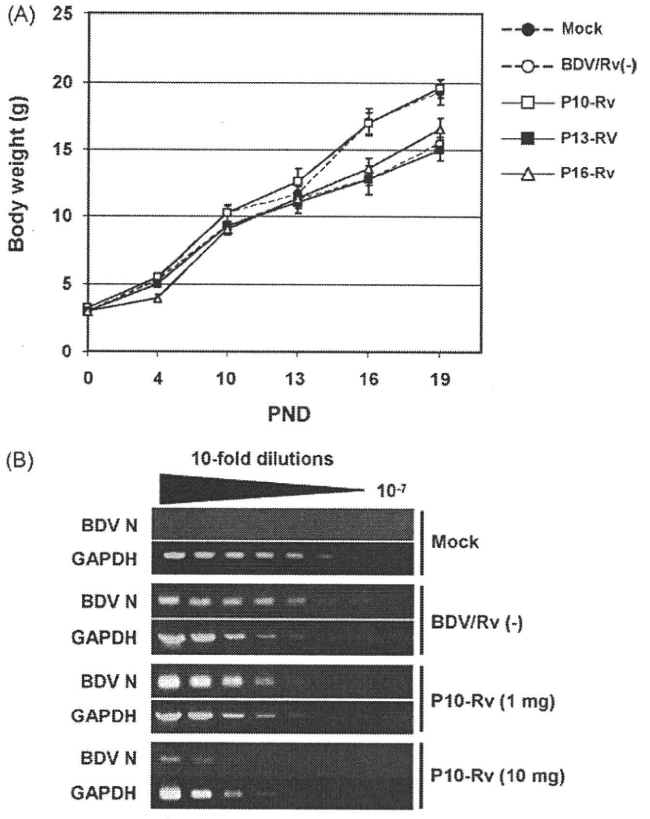

Fig. 1. Ribavirin administration in BDV-infected newborn gerbils. (A) Body weight changes of BDV-infected neonatal gerbils receiving ribavirin at 10 mg/(kg shot) from PD10 (P10-Rv, $n=8$), PD13 (P13-Rv, $n=6$) or PD16 (P16-Rv, $n=4$). Mock, mock-infected newborn gerbil; BDV/Rv(−), BDV-infected, ribavirin-untreated gerbil. Ribavirin was administrated 4, 3 and 2 times in P10-Rv, P13-Rv and P16-Rv gerbils, respectively, by 19 days postinfection. (B) Semiquantitative RT-PCR analysis of BDV N mRNA levels in P10-Rv gerbils. As a control for RNA input, the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was assayed (Lee et al., 2003).

quantitative a RT-PCR for the BDV nucleoprotein (N) region. Total RNA (2 μg) was reverse-transcribed, and the resultant cDNA was amplified with BDV N-specific primers. The analysis revealed that although the expressions of GAPDH are not affected by the treatment, 10 mg/(kg shot) of ribavirin, but not 1.0 mg/(kg shot), clearly reduces the level of BDV RNA expression in the brain (Fig. 1B), suggesting that the propagation of BDV within the brain could be suppressed by the ribavirin treatment.

Previous studies demonstrated that newborn gerbils infected with 200 FFU of BDV developed severe neurological disorders in

Table 1
Summary of ribavirin administration in BDV-infected neonatal gerbils

Ribavirin-treated group[a]	Number of heads	Neurological diseases (%)[b]	Anti-N antibody (%)[c]
NT	10	10(100)	10(100)
P10-Rv	8	1(12.5)	8(100)
P13-Rv	6	2(33.3)	6(100)
P16-Rv	4	2(50)	4(100)

[a] NT: no ribavirin treatment; P10-Rv: ribavirin (10 mg/(kg shot)) treatment from PD10; P13-Rv: ribavirin treatment from PD13; P16-Rv: ribavirin treatment from PD16.

[b] Number of animals that showed signs of neurological disease, including decrease of body weight, paralysis of hind legs, quadriparesis, hypopraxia, debility or blindness.

[c] Number of animals positive for BDV N antibody in serum on the day sacrificed determined by Western blot analysis.

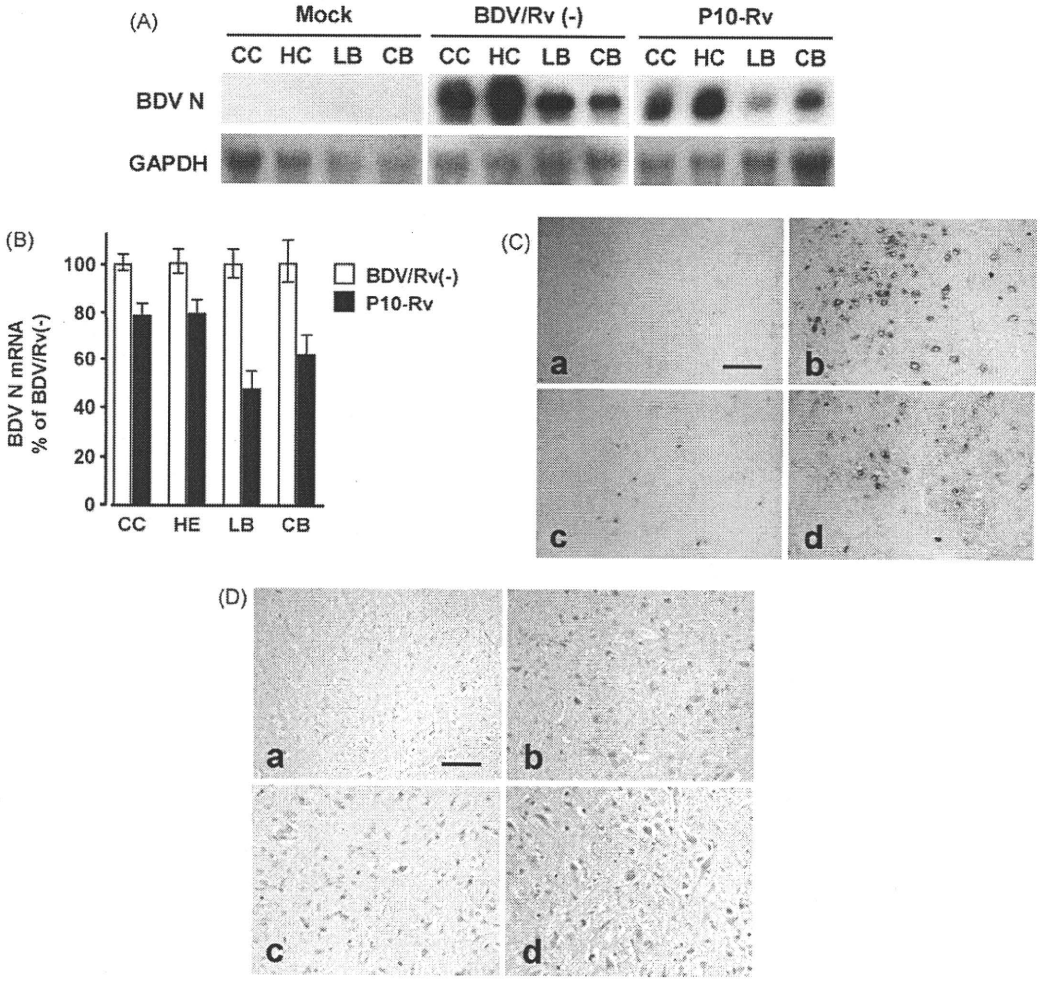

Fig. 2. Reduced proliferation of BDV in ribavirin-treated gerbil brains. (A) Northern blot analysis of P10-Rv gerbil brains. (B) Relative band intensities of N mRNAs in the brains of P10-Rv gerbils. The relative intensities were determined by optical densitometry from Northern blotting shown in (A) (±standard error). CC, cerebral cortex; HC, hippocampus; LB, lower brainstem; CB, cerebellum. (C and D) Expression of BDV replication products in the lower brainstem in neonatally infected gerbils at 25 days postinfection. In situ hybridization (C) and immunohistochemical (D) analyses were performed with an antisense riboprobe specific for the BDV N region and anti-N polyclonal antibody, respectively (Watanabe et al., 2001). Panel a, Mock-infected; b, BDV-infected, untreated; c, P10-Rv; d, P16-Rv. Scale bars; 40 μm.

correlation with significant viral proliferation in specific regions of the brains, including the lower brainstem, by 25 days p.i. (Watanabe et al., 2001). As shown in Fig. 2A and B, the level of viral N mRNA was reduced in all areas examined, especially in the lower brainstem, in P10-Rv gerbils. In situ hybridization using an antisense probe for the N region (Watanabe et al., 2001) revealed that P10-Rv drastically reduced the level of viral RNA in the lower brainstem at PD25 (Fig. 2C). We confirmed the expression level of BDV by an immunohistochemical analysis using rabbit anti-N polyclonal antibody (1:2000 dilution in PBS) generated in our laboratory by using recombinant N antigen (Watanabe et al., 2001). As shown in Fig. 2D, the level of BDV N was also found to be significantly reduced in the brain of P10-Rv gerbils at 25 days p.i. These observations demonstrated that the ribavirin treatment could efficiently repress the proliferation of BDV in the brains of acutely infected newborn gerbil.

A previous study revealed that microglial proliferation is impaired in the brains of BDV-infected, ribavirin-administered rats (Solbrig et al., 2002). To evaluate whether microglial activation is also reduced in the brains of acutely infected gerbils by ribavirin administration, we performed an immunohistochemical analysis using mouse OX-42 monoclonal antibody (1:100 dilution; Serotec; Oxford, UK), which is applicable to the gerbil brains (Hwang et al., 2004). As shown in Fig. 3A, numerous OX-42-positive rod cells were detected in the cortex and brainstem of BDV-infected, untreated gerbils at PD25. On the other hand, P10-Rv gerbils exhibited a marked reduction in the reactivity of OX-42 in the brain. This result suggested that, as reported in a rat model (Solbrig et al., 2002), ribavirin can affect microglial activity even in the acutely infected gerbil brain.

The antiviral activity of ribavirin is shown to be partially due to its ability to enhance Th1 immunity (Liu et al., 1998; Ning et al., 1998). Therefore, we investigated whether the expression of several cytokines, including Th1 cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor α (TNF-α), is enhanced in the ribavirin-treated, BDV-infected newborn gerbils. We performed semiquantitative RT-PCR with primers specific for gerbil cytokines (Lee et al., 2003; Watanabe et al., 2003). As shown in Fig. 3B, expression levels of these cytokines were not shown to increase in the cerebral cortex and lower brainstem by the ribavirin treatment.

In this study, we demonstrated that the ribavirin treatment significantly reduced BDV mRNA in the brains of acutely infected newborn gerbils. Interestingly, although the 10 mg/(kg shot) of ribavirin was fatal in rats (Solbrig et al., 2002), gerbils apparently well-tolerated at the same dose. This may lead to the different effects of ribavirin on BDV replication between the animals. On

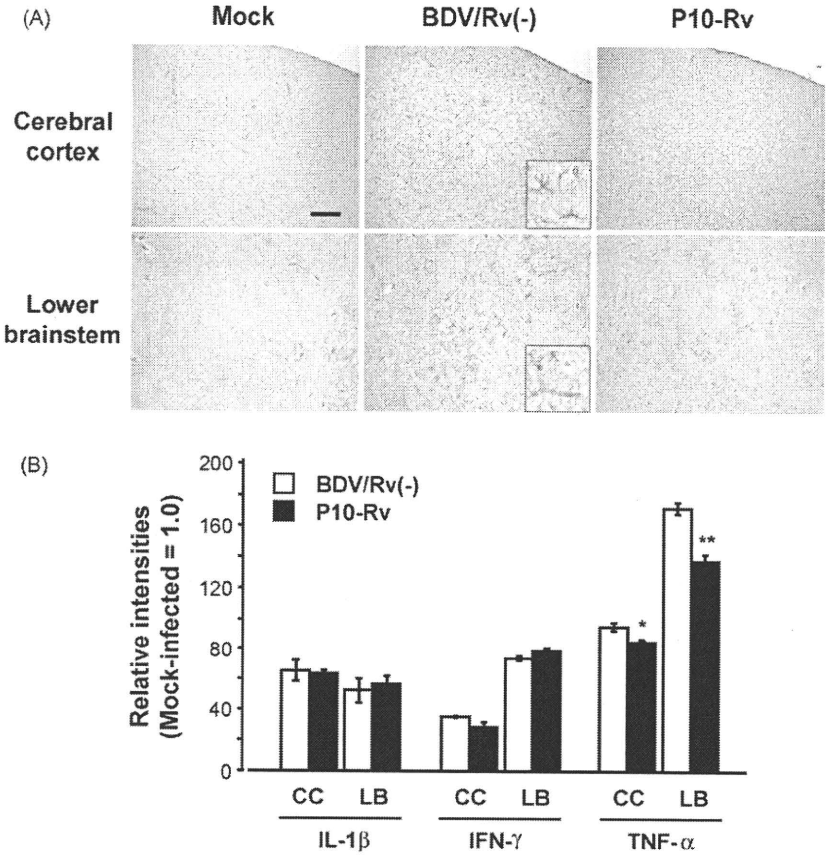

Fig. 3. Ribavirin reduces microglial proliferation, but not cytokine expression, in BDV-infected neonatal gerbils. (A) Reduction in numbers of microglia in the brains of BDV-infected gerbils. Ribavirin administered gerbils were immunostained with mouse OX-42 monoclonal antibody. Mock, mock-infected newborn gerbil; BDV/Rv(−), BDV-infected, ribavirin-untreated gerbil; P10-Rv, BDV-infected gerbil treated with ribavirin at 10 mg/(kg shot) from PD10. Scale bars; 100 μm. At least three sections from at least four animals in each group were investigated. Insets in BDV/Rv(−) show 3× magnificent view of rod-shaped positive cells. (B) Expression of cytokine mRNAs in BDV-infected, ribavirin-treated gerbils. The levels of IL-1β, IFN-γ and TNF-α mRNAs were analyzed with semiquantitative RT-PCR. As a control for RNA input, the level of GAPDH mRNA was assayed (Lee et al., 2003). The amplification products were resolved on 1.5% agarose gels and transferred onto nylon membranes. The membranes were then analyzed by Southern blot hybridization with digoxigenin-labeled specific probes for gerbil cytokines (Watanabe et al., 2003). The relative intensities to that of mock-infected gerbils were indicated. The intensity of each band on X-ray films was quantified using NIH image. Values were expressed as means ± standard error IL-1β: CC [BDV/Rv(−): 65.44 ± 7.08; P10-Rv: 63.38 ± 2.52], LB [BDV/Rv(−): 52.08 ± 7.99; P10-Rv: 56.46 ± 5.19], IFN-γ: CC [BDV/Rv(−): 35.05 ± 0.81; P10-Rv: 28.39 ± 2.87], LB [BDV/Rv(−): 73.78 ± 1.54; P10-Rv: 78.89 ± 1.62], TNF-α: CC [BDV/Rv(−): 95.25 ± 2.42; P10-Rv: 84.90 ± 1.64], LB [BDV/Rv(−): 171.56 ± 3.45; P10-Rv: 137.67 ± 4.07)]. $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). CC, cerebral cortex; LB, lower brainstem.

the other hand, it is likely that the ribavirin enters into systemic circulation of the gerbils, because we administrated a 10 μl of ribavirin solution into the subarachnoid cavity of the animals. This might partially induce the difference in the susceptibility between the rats and gerbils. Our study revealed that the administration of ribavirin from PD10, when the BDV has not yet proliferated throughout the brain, could efficiently inhibit the viral expansion within the CNS. In previous studies (Watanabe et al., 2001, 2003), we showed that the expression areas of BDV mRNA and proteins in the brains are shifted in association with disease progression and that the disease onset may be correlated with BDV propagation within the brainstem region. Thus, the ribavirin treatment may prevent BDV to reach the brainstem of P10-Rv gerbils.

We previously concluded that both direct damage by BDV propagation and an indirect effect of the cytokines, such as IL-1β, are required for induction of fatal neurological disorders in gerbils (Watanabe et al., 2003). We found that the ribavirin treatment does not change the expression level of IL-1β, indicating that only an abnormal level of IL-1β is not enough to induce neurological disorders in the gerbils. On the other hand, the level of TNF-α was significantly decreased in both the cerebral cortex and lower brainstem regions of ribavirin-treated gerbils. It would be of interest to investigate the effect of TNF-α on the induction of the neurological symptoms in BDV-infected gerbils.

Ribavirin is known to exert antiviral effects through the following mechanisms; (i) interference with the capping of viral mRNAs (Bougie and Bisaillon, 2004), (ii) inhibition of the polymerase activity of viruses (Crotty et al., 2000) and (iii) induction of error catastrophe in viral genomes (Crotty et al., 2001; Severson et al., 2003). Previous reports have clearly showed that transcription of BDV is inhibited by ribavirin treatment in persistently BDV-infected cell lines, such as human oligodendrocytes and rat glioma cells (Jordan et al., 1999; Mizutani et al., 1998). These studies suggested that a reduction in the size of the intracellular GTP pool could be a mechanism for inhibition of the transcription and capping of BDV mRNA. The significant reduction of microgliosis suggested that the depletion of the GTP pools actually occur in the gerbil brain cells. On the other hand, despite the reduced activation of microglial cells by the ribavirin treatment, the expression levels of the cytokines in the treated gerbils appeared to be comparable to the untreated animals. Considering the ability of ribavirin to enhance Th1 immunity, it is conceivable that the ribavirin treatment enhances the expression of the cytokines from the other resident cells in the brains, such as astrocytes. Further studies will need to elucidate the sources of the cytokine expression in the ribavirin-treated animal brains.

In this study, we demonstrated that ribavirin could directly reduce viral propagation within the brains of acutely infected newborn gerbils. Our results indicate that ribavirin might be an effective tool for research into the replication and pathogenesis of BDV in vivo, as well as in the discovery of an antiviral strategy against infections of the CNS.

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Isotype analysis of human anti-Borna disease virus antibodies in Japanese psychiatric and general population

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ABSTRACT

Background: Borna disease virus (BDV) infection of humans has not been widely accepted due to the low titer of the antibody and lack of reliable diagnostic tools.
Objectives: To examine exposure to BDV or a related virus in Japanese psychiatric and general population by detecting the specific IgG, IgM, and IgA.
Study design: Sera from 304 psychiatric and 378 control subjects were examined for IgG, IgM, and IgA against BDV nucleoprotein (N) and phosphoprotein (P) using highly specific and sensitive radioligand assay. The avidity was also examined.
Results: The specific IgG, IgM, and IgA against both BDV-N and -P were detected and the seropositivity was not significantly different between patients and controls. The avidity of the specific IgG was low to moderate, and the specific IgM did not disappear for several years.
Conclusions: Our results suggested common exposure to BDV or a related virus in the general Japanese population. Low avidity IgG and persistent IgM suggested delayed immune response against BDV or a related virus. The specific IgA indicated mucosal involvement.

1. Introduction

Borna disease virus (BDV), a pathogenic agent for meningoencephalitis in horse in Germany, has been found to cause central nervous system disease in a variety of vertebrate species, having broad geographic distribution.[1,2] Its possible infection in humans has not been established. One reason would be the low titer of the specific antibodies. The antibodies have been detected using several methods, but the titers were as low as 1:5–1:40, the avidity was persistently low,[3] and the results did not agree among different methods or laboratories. Therefore, the possibility of cross-reaction has been proposed.[2,4] However, it was reported that human sera had antibodies against a variety of epitopes of BDV antigens, indicating that these antibodies would have been produced by exposure to BDV or a closely related agent.[5] Recently, we have detected anti-BDV IgG using a highly sensitive and specific radioligand assay.[6] Now, we have developed the method to detect the specific IgM and IgA and measured them in large numbers of psychiatric and control subjects.

2. Subjects and methods

2.1. Serum specimens

The profiles of the subjects are shown in Table 1. All psychiatric samples were taken from patients at the Department of Psychiatry, Osaka General Medical Center. Two hundred consecutive samples from blood donors in Osaka were obtained without any personal information. Seventy-six healthy controls were obtained from volunteers with no abnormalities for routine blood analysis. One hundred and two cord blood samples were taken consecutively in Osaka Medical Center for Maternal and Child Health. Each Medi-

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Table 1
Profiles of the subjects

	n	Sex (M/F)	Age
Psychiatric patients	304	98/206	46.2 ± 16.3
Schizophrenic disorders	91	28/63	43.8 ± 14.4
Mood disorders	129	38/91	49.5 ± 16.4
Other psychiatric disorders	84	32/52	43.6 ± 17.2
Control subjects	276		
Blood donors	200	Unknown	Unknown
Healthy subjects	76	Unknown	Unknown
Cord blood subjects	102	0/102	29.0 ± 4.3

cal Center's Ethical Committee approved this research, and written informed consent was obtained from the patients.

2.2. Radioligand assay

2.2.1. Antigens

The radio-labeled BDV-N and -P proteins were produced by incubating BDV-N/pET28a or BDV-P/pET28a and [^{35}S]methionine (Amersham Biosciences, Piscataway, NJ) in TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) at 30 °C for 90 min. The detailed information for BDV-N/pET28a and BDV-P/pET28a was described previously.[6] The product was applied to Nick column (Amersham Biosciences) with reaction buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1% Tween-20, and 0.1% NaN_3, pH 7.4) to remove free [^{35}S]methionine.

2.2.2. Assay procedure

The assay procedure was modified from our previous report.[6] Thirty microliters of the reaction buffer was put into each well of a 96-well filtration plate (Millipore, Bedford, MA). Then, 2 μl of sera in duplicate, 10 μl of 25% Protein G Sepharose (Amersham Biosciences) diluted with reaction buffer, and 10 μl of the labeled BDV protein containing 20 kcpm reactivity diluted by reaction buffer were added. The filtration plate was incubated overnight at 4 °C under gentle mixing. The liquid was removed using a Vacuum Manifold (Millipore), and the Protein G Sepharose in the well was washed with 200 μl of washing buffer (50 mM Tris–HCl, 150 mM NaCl, and 1% Tween-20, pH 7.4) four times for 60 min. The filtration plate was centrifuged at 1500 g for 1 min and dried. Then, 35 μl of OptiPhase SuperMix (PerkinElmer Life Science, Boston, MA) was added, and the precipitated labeled protein was counted in 1450 MicroBeta (PerkinElmer, Wellesley, MA). To detect the specific IgM and IgA, agarose-labeled anti-human IgM (μ-chain specific, goat IgG; Sigma, Saint Louis, MO) or IgA (α-chain specific, goat IgG; Sigma, Saint Louis, MO) was used in place of Protein G Sepharose.

2.2.3. Calculations

As a positive control for anti-BDV-N and -P IgG we used serum no. 199-5 with the highest titers, diluted 10-fold with a negative serum, and its index values were decided as 10.0 and 7.0, respectively, in order to match with those in our previous method. As for the specific IgM, serum no. 140-2, moderately positive for BDV-N and -P IgM, was used. For IgA, serum no. 246, highly positive for both antigens diluted 10-fold with a negative serum, was used. The index values were decided as 6.3 and 2.7 for BDV-N and -P IgM, and 4.0 and 3.0 for anti-BDV-N and -P IgA, respectively.

The mean + 4S.D. of the samples excluding apparently positive samples were employed as cut-off points. The average intra-assay coefficients of variation were 2.8% (from 0.5% to 9.9%), 6.1% (from 1.2% to 9.5%), and 5.2% (from 1.2% to 10.8%) for IgG, IgM, and IgA, respectively, and the average inter-assay coefficients of variation were 8.0% (from 2.3% to 14.9%), 13.9% (from 4.9% to 24.1%), and 23.8% (from 7.6% to 38.5%), respectively.

2.3. Avidity examination

The avidity of the specific antibodies was examined by incubating them in 3 and 5 M urea in PBS for 3 min during the washing procedure.

3. Results

The specific IgG against BDV-N and -P were found in samples from all groups (Fig. 1, Table 2). There were no significant differences between groups and subgroups except between schizophrenic disorders and other psychiatric disorders for anti-BDV-P IgG (3/91 (3.4%) and 12/84 (14.3%), respectively; $p = 0.0095$, χ^2 test). The specific IgM and IgA were found in samples from the psychiatric and control groups but not in cord blood samples (Fig. 1, Table 2), and there were no significant differences between any groups or subgroups except cord blood subjects.

In the psychiatric subjects, the rate of having at least one specific antibody did not differ significantly between sex (male and female: 19/98 (19.4%) and 51/206 (24.8%), respectively; $p = 0.30$, χ^2 test), whereas elder patients aged 51 or over had a significantly higher positive rate than younger patients (younger and elder: 34/181 (18.8%) and 36/123 (29.3%), respectively; $p = 0.033$, χ^2 test). Examined for each antibody, the rate of having anti-BDV-N IgG was significantly different between younger and elder patients (7/181 (3.9%) and 17/123 (13.8%), respectively; $p = 0.0080$, χ^2 test), whereas the rates of having the other antibodies were not significantly different between the age groups.

Three subjects had 4 specific antibodies, 2 subjects had 3 antibodies, and 39 had 2 antibodies (Table 3).

Table 2
Number of subjects having anti-BDV antibodies

	n	Anti-N IgG	Anti-P IgG	Anti-N IgM	Anti-P IgM	Anti-N IgA	Anti-P IgA	Number of co-existing antibodies				
								One	Two	Three	Four	At least one
Psychiatric patients	304	25	27	8	10	12	8	53	15	1	1	70 (23.0%)
Schizophrenic disorders	91	9	3*,**,***	4	2	3	2	19	2	0	0	21 (23.1%)
Mood disorders	129	8	12**	3	6	6	2	16	7	1	1	25 (19.4%)
Other psychiatric disorders	84	8	12***	1	2	3	4	18	6	0	0	24 (28.6%)
Control subjects	276	24	27*	13	9	13	10	43	21	1	2	67 (24.3%)
Blood donors	200	17	16	9	8	11	7	29	14	1	2	46 (23.0%)
Healthy subjects	76	7	11	4	1	2	3	14	7	0	0	21 (27.6%)
Cord blood subjects	102	6	7	0	0	0	0	7	3	0	0	10 (9.8%)

There were no significant differences between psychiatric patients and control subjects for any antibodies. The p values for the rates of positive anti-BDV-P IgG between schizophrenic disorders and other groups are shown. * $p = 0.0502$; ** $p = 0.082$; *** $p = 0.0095$.