diseases *in vitro*, and the procedure of extracting various cytokines from bronchial ELF reported by Ishizaka et al[8,9] was used as the standard procedure: (1) sampling time was 30 s; (2) the probe was cut and placed in a tube containing saline solution; (3) the probe was stirred for 60 s using a vortex to dislodge bacteria adsorbed to the probe; and (4) 1/10 serial dilutions were prepared and subjected to colony counting. The ratio of the amount of bacteria sampled by the standard BMS procedure to the amount in suspension for sampling was calculated, and the value was almost constantly 1 to 3%, indicating that when 33 to 100 bacterial cells are present per 1-mL sample, one bacterium can be detected using BMS. Since $\geq 1 \times 10^4$ cfu/mL bacteria are present in BAL from patients with VAP collected using a bronchoscope, bacteria are theoretically detectable using BMS.[1,18]

Regarding the relationship between the amounts of bacteria and water collected by BMS, interestingly, the amount of bacteria was proportional to the collectable water amount. In BMS, bacteria are adsorbed together when water is adsorbed to the BMS probe. According to Ishizaka et al,[8,9] one probe adsorbs 2 to 20 μL, which corresponds to 0.2 to 2% of 1 mL, and the sampling rate of approximately 1 to 3% in our experiments was consistent. BMS is capable of collecting 20 μL of bronchial ELF and all bacteria in the ELF, which may contain mucus and pus depending on the local lesion. Thus, detection using BMS is sufficiently quantitative. Regarding the sampling rate of each bacterial species, no significant difference was noted among *S pneumonia, H influenzae*, and MAC, but the *P aeruginosa* sampling rate was significantly higher than the other three species. Although the reason was not clarified, *P aeruginosa* may easily adhere to polyethylene tubes.

The amount of bacteria for sampling, sampling time, stirring time, and use of surfactant were investigated by modifying the standard procedure using the four bacterial species. Regarding the bacterial amount for sampling, when the suspension was diluted to 1/100, the sampling rate did not change, showing that the bacterial amount in suspension does not affect the sampling rate using BMS. When the sampling time was changed from 30 to 5 s, and the stirring time was changed from 60 to 10 s, the sampling rate was not affected, indicating that bacteria are easily dislodged from the probe. A surfactant was added during stirring to investigate its effect on bacterial recovery, but the sampling rate was not changed compared to stirring in saline solution alone. Thus, the shortened standard procedure may provide equivalent results and make the procedure more easily applicable in clinical practice.

Investigation of storage conditions (standing time) after sampling is also important. Transport of samples to the site of bacterial testing may often take time in clinical practice. When the probes after sampling were kept in tubes containing saline solution for 3 h, the bacterial amounts of *S pneumoniae, H influenzae, P aeruginosa*, and MAC were not changed compared to those processed by the standard procedure, clarifying that processing of samples within 3 h after sampling is acceptable for clinical practice. Although we did not investigate storage overnight or at 4°C, according to Forceville et al,[19] when bacteria in bronchoscopic samples were quantified after storage at 4°C for 48 h, no changes were noted in the amounts of Staphylococcus, Enterobacteriaceae, and Pseudomonas, showing that storage is possible, but Haemophilus decreased after storage. Since Haemophilus easily perishes at low temperature, and storage conditions vary among species, the understanding of individual bacterial characteristics is necessary to evaluate the results.

The sampling rates using PSB and BMS were compared *in vitro*. The usefulness of PSB for diagnosis of respiratory infections has been reported.[1] PSB is similar to BMS in the following ways: the brush is protected by a sheath and comes out of the sheath only during sampling, avoiding contamination with oral indigenous bacteria, and bacteria are directly collected from the lesion. In the experiment using PBS, the sampling rate was not constant among the bacterial species, and the *P aeruginosa* sampling rate was markedly high. On comparison with BMS, the recoveries of *S pneumoniae, H influenzae*, and MAC using PBS were significantly lower than those using BMS, but the recovery of *P aeruginosa* was similar. Since only bacteria adhered to the PBS brush are recovered when bacteria are directly sampled from bacterial suspension, the amount of the three bacterial species collected using PBS were naturally lower those collected using BMS, but *P aeruginosa* was highly adherent to the brush. The sampling rate may be higher when purulent secretion is collected from local lesions of infection.

This *in vitro* study clarified that BMS is capable of the quantitative sampling of bacteria. Further studies are necessary to clarify its applicability as a diagnostic device, as have been performed for PSB and BAL, which have been applied to clinical cases and reported to be useful.

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Hypersensitivity Pneumonitis Caused by *Penicillium citrinum*, not Enoki Spores

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Background *Flammulina velutipes is called the Enoki mushroom in Japanese and is cultivated indoors. Mushroom workers face occupational exposure to a tremendous number of fungi and organic antigens capable of causing hypersensitivity pneumonitis (HP). One worker employed at an Enoki farm developed HP due to Penicillium citrinum. This study investigated new cases of HP among the workers cultivating Enoki.*

Methods *Serum Krebs von der Lungen-6 (KL-6), surfactant protein (SP)-A and SP-D were measured. Lymphocyte stimulation tests (LST) and double immunodiffusion tests (DIT) were performed to identify P. citrinum. Workers showing high levels of KL-6, SP-A, or SP-D and a high LST value or positive DIT were identified and then were further examined by chest computed tomography, bronchoalveolar lavage and transbronchial lung biopsy. The initial patient and new HP patients were defined as the HP group and the other participants were defined as the non-HP group.*

Results *Forty-eight Enoki workers participated in the study. Four of nine workers who met the criteria for further examinations were diagnosed as having HP due to P. citrinum. In comparison between non-HP group and HP group, KL-6, SP-D and LST values were significantly higher in HP group. There was a strong correlation between KL-6 and SP-D. DIT had high sensitivity and high specificity.*

Conclusions *KL-6, SP-D, LST, and DIT were useful for detecting HP patients. KL-6 was the most useful predictor of HP in this study. DIT was useful not only as a predictor of HP but also as a detector of the causative antigen.* Am. J. Ind. Med. 50:1010–1017, 2007.

KEY WORDS: hypersensitivity pneumonitis; KL-6; double immunodiffusion test; lymphocyte stimulation test; Penicillium citrinum

INTRODUCTION

Hypersensitivity pneumonitis (HP) is an allergic immunoreactive disease caused by the inhalation of a variety of environmental agents. HP among mushroom workers is called mushroom worker's lung (MWL). Mushroom workers face occupational exposure to a tremendous number of fungi, bacteria, and organic antigens capable of causing HP, and some workers in fact, do develop HP. The onset of MWL is generally during the first few months of employment, but it sometimes occurs in workers who have been employed for many years [Sanderson et al., 1992]. Some outbreaks of HP have been reported among mushroom workers [Steawart, 1974; Sanderson et al., 1992; Mori et al., 1998]. The clinical features of MWL are similar to those of HP. Patients commonly present with symptoms of nonproductive cough, shortness of breath, low grade fever and malaise. These symptoms mimic the so-called common cold. General clinical symptoms and laboratory data including white blood

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Accepted 19 September 2007
DOI 10.1002/ajim.20535. Published online in Wiley InterScience (www.interscience.wiley.com)

count, are not sufficient to detect this HP [Tsushima et al., 2001].

The most common type of MWL is caused by the inhalation of a large number of *Thermophilic actinomycetes* [Van den Bogart et al., 1993] from the culture medium used for the cultivation of *Agaricus bispurus* [Sanderson et al., 1992] in Europe. In Japan, however, MWL caused by spores of mushrooms such as *Cortinus shiitake* [Matsui et al., 1992], *Pholiota nameko* [Nakazawa and Tochigi, 1989], *Lyophyllum aggregatum* [Tsushima et al., 2001], and *Pleurotus eryngii* [Saikai et al., 2002] have been recently reported. There have been no previous cases reported of MWL caused by spores of *Flammulina velutipes*, which is known as the Enoki mushroom in Japanese. Enoki mushrooms are one of the most popular mushrooms in Japan, and they are cultivated indoors to produce large quantities year round.

A 47-year-old Japanese woman, who had been engaged in Enoki production for 22 years, developed HP [Yoshikawa et al., 2006]. Precipitins found in her serum were positive to *Penicillium citrinum* but not to Enoki spores, and *P. citrinum* was considered the most likely antigen. *Penicillium* spp. is widespread and found in soil, decaying vegetation and in the air. More than 150 different species have been identified in the genus *Penicillium* [Pit, 1998], some of which are known to be capable of causing various forms of HP such as humidifier/air conditioner lung [Solley and Hyatt, 1980], farmer's lung [Nakagawa-Yoshida et al., 1997] and HP in the home [Fergusson et al., 1984; Park et al., 1994]. *P. citrinum*, one of these species, is commonly found indoors, and produces flat green colonies. Wei et al. [1993] reported that about 40.5% of the *Penicillium* colonies isolated from the Taipei urban area were *P. citrinum*. Licorish et al. [1985] reported that *Penicillium* spores in relatively natural states and numbers were potent asthma immunopathogens. However, there have been no previous reports of HP caused by *P. citrinum*. A study was conducted among the remaining workers at the mushroom farms in the rural area to find new cases of HP caused by *P. citrinum*. Tsushima et al. [2005] reported that serum Krebs von der Lungen-6 (KL-6) and surfactant protein (SP)-D may be related to the resolution of HP in addition to lymphocyte stimulation test (LST) values and chest CT. Some articles reported that the serum KL-6 is a good marker for disease activity in interstitial pneumonitis such as HP [Kohno et al., 1989; Kobayashi and Kitamura, 1995]. SP-A and SP-D are also known to be good markers indicating disease activity of HP [Tanaka et al., 2000]. These biomarkers were initially used to identify the suspected HP patients.

MATERIALS AND METHODS

All examinations were approved by the Research Committee of the Shinshu University School of Medicine. Written informed consent was obtained from all participants.

Methods

Enoki mushrooms were grown in small closed rooms where air and moisture conditioners were equipped to maintain a suitable temperature and humidity. In eastern Nagano prefecture, 57 workers were employed to cultivate Enoki mushrooms at 18 different farms. They were asked to voluntarily participate in this study which included a questionnaire, culture of the nasal cavity, serologic testing, LST [Holland and Mauer, 1964] and double immunodiffusion test [DIT, Ouchterlony's immunodiffusion test, Ouchterlony, 1957]. All enrolled workers worked until the same day when blood samples and a culture of the nasal cavity were obtained. The workers were divided into three working groups: (1) growing/maintenance; (2) picking; and (3) packing. The growing/maintenance department workers prepared and evaluated the culture medium, and checked environmental conditions including temperature and moisture. Pickers cut the mushrooms from the containers and packers packaged the harvested mushrooms.

Diagnosed HP patients and the initial patient were defined as the HP group and the other participants were defined as the non-HP group. In further examinations, cases without HP were also defined as the non-HP group. Four males and four females (mean age, 49.6 years old; range, from 43 to 57 years old) who had never been engaged in Enoki mushroom production were enrolled as controls. Blood samples were obtained from them for LST and DIT.

Questionnaire

The questionnaire obtained information on each worker's age, gender, smoking history, allergic history, employment history, and respiratory symptoms. Information was obtained by a self-reporting questionnaire, and blood samples were obtained immediately after the questionnaire and informed consent was completed.

Culture of Nasal Cavity

Culture specimens were obtained from the nasal cavity of each worker. The specimen was cultured on both Sabouraud agar and potato-dextrose agar (PDA) to detect *Penicillium* spp. Mycological identification of *Penicillium* spp. was conducted by the Japan Agricultural Cooperative Association in Nagano prefecture.

Serologic Testing

KL-6, SP-A, and SP-D were examined on each worker. KL-6 was measured at the Clinical Laboratory Department of Shinshu University using an electrochemiluminescence immunoassay (Picolumi®KL-6). The limit of detection for KL-6 was 51 U/ml, and the cut-off level was determined to be

450 U/ml based on the data of Yokoyama et al. [1998]. SP-A and SP-D were measured at a laboratory (SRL: Special Reference Laboratory, Inc., Tokyo, Japan) using an EIA. The limits of detection for SP-A and SP-D were 1.1 and 17.3 ng/ml, respectively, and the cut-off levels were determined using SRL research to be 43.8 and 110 ng/ml, respectively.

Lymphocyte Stimulation Test (LST)

LSTs to *P. citrinum*, Enoki, and the culture medium, composed of six types of organic matter (rice sediment, wheat bran, bean husks, bean curd refuse, corn dregs, and beet dregs) in sera were performed in all participants and normal controls at SRL without information about their clinical histories. *P. citrinum*, Enoki and the culture medium were provided by the Japan Agricultural Cooperative Association in Nagano prefecture. The method has been described previously [Yoshikawa et al., 2006]. Enoki solution and the culture medium solution were diluted to 50-, 250-, 1,250-, 6,250-, 31,250-, and 156,250-fold. The *P. citrinum* solution was diluted to 1,500, 300, 60, 12, 2.4, and 0.48 spores/μl. LST values were calculated using the formula [Tsushima et al., 2001].

Double Immunodiffusion Test (DIT) (Ouchterlony's Immunodiffusion Test)

Double immunodiffusion tests to *P. citrinum*, Enoki and the culture medium in sera were performed on all participants and normal controls to detect precipitating antibodies at our laboratory. The antigen preparation and method have been previously described [Yoshikawa et al., 2006]. The concentration of each antigen solution was adjusted to 10 mg/ml. To confirm that this assay system worked well, Bunashimeji mushroom antigen and serum, from a patient with HP due to Bunashimeji mushroom, were used as a positive control.

Further Examinations

Methods (the criteria for further examination)

Workers with KL-6 ≥450 U/ml, SP-A ≥43.8 ng/ml, or SP-D ≥110 ng/ml and with a positive response to *P. citrinum* on LST or DIT were identified and examined further by CXR, chest computed tomography (CT), bronchoalveolar lavage (BAL), and transbronchial lung biopsy (TBLB). All selected workers worked at the mushroom farms immediately before undergoing further examinations.

CXR and chest CT

CXR and chest CT were obtained from selected workers. Chest CT scanning (HiSpeed Advantage; GE Medical Systems; Milwaukee, WI) was performed with standard 10-mm-thick contiguous scanning. CXR and Chest CT images were reviewed by two chest radiologists who had no knowledge of the workers' clinical findings.

BAL fluid (BALF) and TBLB

BAL was performed following our laboratory method [Kubo et al., 1998]. A portion of the obtained cells was used for counting the total cell numbers using a hemocytometer and for analyzing lymphocyte subsets by flow cytometry using CD4 and CD8 monoclonal antibodies (Becton Dickinson Co; Mountain View, CA). Cell spreads prepared by cytocentrifugation were stained with May-Giemsa stain. A portion of the obtained cells was also used for the LST to *P. citrinum*, Enoki and the culture medium. Part of the supernatant fluid was used for culture. TBLB was also performed on selected workers. TBLB was usually performed in lobes which had abnormal shadows. If there were no abnormal shadows observed in the chest CT scan, specimens were obtained from lobes within the right lung, if possible. In this study, at least three biopsies were obtained by fluoroscopic guidance from lobes within a single lung, and evaluated with hematoxylin eosin (HE) stain.

The diagnosis criteria of HP

For the diagnosis of HP due to *P. citrinum*, the following diagnostic criteria were used: clinical features such as cough, shortness of breath; a reticulonodular shadow on CXR and/or CT scans; predominant lymphocytosis in BALF; lymphocyte infiltrate in alveolitis with granuloma formation on pathological specimens; positive serum precipitin findings; a positive LST reaction to *P. citrinum*; and resolution of episodic respiratory symptoms after exposure to *P. citrinum* ceased [Tanaka et al., 2001].

Statistical analysis

All values given in the text and tables are expressed as the mean ± standard deviation (SD). The Wilcoxon/Kruskal–Wallis tests for independence were used for comparisons between the groups. A P-value of <0.05 was considered to be significant.

RESULTS

Forty-eight of 57 workers (23 males and 25 females) were enrolled in this study. Subjects ages ranged from 31 to 74 years (mean, 53.1 years), and the duration of working ranged from 3 months to 40 years (mean, 18.4 years). There were 13 smokers and 7 ex-smokers, while 28 subjects had never smoked. Six workers had allergic histories such as atopic dermatitis or hay fever. All 48 workers were involved

with picking and/or packing, and 12 workers were involved with a variety of responsibilities related to growth/maintenance. Nine workers complained of a dry cough, shortness of breath and/or low grade fever. Three of the 9 workers reported acute symptoms consistent with HP. On the nasal cavity culture, *P. citrinum* was isolated from 54.2% of the workers, while Enoki was isolated from only 8% of the workers (Table I).

In blood samples, KL-6 levels ranged from 152 to 6,683 U/ml (mean, 516.3 U/ml), and were positive in seven workers (14.6%). SP-A levels ranged from 9.7 to 108 ng/ml (mean, 33.7 ng/ml), and were positive in five workers (10.4%). SP-D levels ranged from 17.2 to 437 ng/ml (mean, 68.4 ng/ml), and were positive in five workers (10.4%) (Table II).

LST values exceeding 200% are considered to be positive according to SRL. However, LST values to *P. citrinum* of normal controls ranged from 198% to 360% (mean, 284.4%) in this study. The cut-off level was finally decided to be 400% based on the data of normal controls. In the same way, LST values to Enoki and the culture medium were examined. LST values to Enoki and the culture medium of normal controls ranged from 267% to 380% (mean, 330%) and from 185% to 346% (mean, 281%), respectively. The cut-off levels in Enoki and the culture medium were finally decided to be both 400%. LST revealed a high positive rate for each antigen. LST values to *P. citrinum*, Enoki, and the culture medium were positive in 22 (45.8%), 26 (54.2%), and 26 (54.2%) workers, respectively. Precipitating lines were observed to *P. citrinum* in five workers (10.4%) in DIT. There were no precipitating lines observed to Enoki or culture medium in any of the workers. Normal controls had no precipitins to either *P. citrinum*, Enoki or the culture medium (Table II).

TABLE I. Characteristics of the 48 Enoki Workers

	Total	Male	Female
Subjects	48	23	25
Age (years old)	53.1 ± 10.6	53.3 ± 10.4	52.9 ± 11.1
History of allergy	6 (12.5%)	2 (8.7%)	4 (16%)
Smoking history			
Current-smoker	13 (27.1%)	12 (52%)	1 (4%)
Ex-smoker	7 (14.6%)	4 (17.4%)	3 (12%)
Never-smoked	28 (58.3%)	7 (30%)	21 (84%)
Duration of working (years)	18.4 ± 9.6	19.3 ± 8.4	17.5 ± 10.7
Symptoms	9 (18.8%)		
Dry cough and sputum	6 (12.5%)	1 (4.3%)	5 (20%)
Shortness of breath	4 (8.3%)	2 (8.7%)	2 (8%)
Fever	1 (2.1%)	0 (0%)	1 (4%)
Culture of nasal cavity	26 (54.2%)[a]	13 (56.5%)	13 (52%)

[a]Percentage of workers from whom *Penicillium citrinum* was isolated.

Further Examinations

Nine workers met the criteria for further examinations. The subjects consisted of 3 males and 6 females, and 6 workers were working as pickers and/or packers, and 3 workers were engaged in everything. Six of the 9 workers demonstrated respiratory symptoms such as dry cough, shortness of breath, and low grade fever. Shortness of breath was very severe in cases 1, 8, and 9, and their Fletcher-Hugh-Jones levels ranged around III–IV. In cases 1, 6, 8, and 9, respiratory symptoms resolved within several days of being away from the work environment, and then reappeared gradually after they returned to work again. CXR demonstrated diffuse ground glass opacity (GGO) in both lung fields in cases 1, 8, and 9, and their chest CT images showed centrilobular small nodule as well as GGO. In case 6, there was no abnormal shadow on the CXR, but the chest CT demonstrated bilateral diffuse GGO (Table III).

Total cell counts in the BALF ranged from 11.9×10^4 to 309×10^4 (mean, 67.3×10^4), and the percentage of lymphocytes ranged from 13.2% to 91.5% (mean, 57.2%). The CD4/CD8 ratio of the lymphocyte surface markers ranged from 0.3 to 2.5 (mean, 1.26). Specimens obtained by TBLB showed lymphocyte infiltrated alveolitis in cases 1, 6, 8, and 9, and a Masson body in case 9. There was no epitheloid noncaseating granuloma in specimens obtained from any of the workers examined. In the BALF obtained from case 6, *Penicillium* spp. was cultured on PDA but it was not possible to identify the species (Table IV).

LST in BALF was performed to *P. citrinum*, Enoki and the culture medium. The LST values in BALF to *P. citrinum* were positive in cases 1, 5, 6, and 9 though most cases had positive reactions to *P. citrinum* on LST in sera. In case 9, the LST value to *P. citrinum* was negative in serum but positive in BALF. Positive serum precipitin findings to *P. citrinum* were shown in cases 1, 6, 8, and 9 (Table V). Although no inhalation challenge to *P. citrinum* was performed in the 9 selected workers, cases 1, 6, 8, and 9 were finally diagnosed as having HP caused by *P. citrinum* based on the diagnostic criteria of HP.

Biomarkers were compared among workers (Table VI). KL-6 and SP-D in sera were significantly higher in the HP group ($n = 5$) than those in the non-HP group ($n = 44$, $P = 0.0004$, $P = 0.0037$, respectively). There was no significant difference in SP-A between the non-HP group and the HP group. The LST values in sera to *P. citrinum* were significantly higher in the HP group than those in the non-HP group ($P = 0.011$). The LST values in BALF to *P. citrinum* were also examined in the 9 selected workers and the initial HP patient (non-HP group, $n = 5$ vs. HP group, $n = 5$). The LST values were significantly higher in the HP group ($2,772 \pm 2,124.2$) than those in the non-HP group (203 ± 213.4, $P = 0.027$). The area under the curve (AUC) in a receiver operating characteristic (ROC) analysis was the

TABLE II. Laboratory Data of the Blood Samples From the 48 Enoki Workers

	Average		Positive (%)
KL-6 (U/ml) (≥450 U/ml)	516.3 ± 1,096.4		7/48 (14.6)
SP-A (ng/ml) (≥43.8 ng/ml)	33.7 ± 16.5		5/48 (10.4)
SP-D (ng/ml) (≥110 ng/ml)	68.4 ± 82.0		5/48 (10.4)
	LST		
	LST value (%)	Positive (%)[a]	Double immunodiffusion test (%)
Penicillium citrinum	497.8 ± 584.9	22/48 (45.8)	5/48 (10.4)
Enoki	652.8 ± 658.5	26/48 (54.2)	0/48 (0)
Culture medium	554.9 ± 421.0	26/48 (54.2)	0/48 (0)

KL-6, Krebs von der Lungen-6; SP-A, surfactant protein-A; SP-D; surfactant protein-D; LST, lymphocyte stimulation test.
[a]LST value ≥400% is considered to be positive on each antigen.

best of 0.99 for KL-6 when the cut-off level was 450 U/ml. The AUC for SP-D was as good as 0.90 when the cut-off level was 110 ng/ml. The AUC in LST was 0.87 when the cut-off level was 400%. The sensitivity and specificity of KL-6 showed 100% and 93.2%, respectively. The sensitivity and specificity of DIT showed 100% and 97.5%, respectively. In the LST, the sensitivity was as good as 80%, but the specificity was as low as 56.8%.

DISCUSSION

In this study, workers were selected showing KL-6 ≥450 U/ml, SP-A ≥43.8 ng/ml or SP-D ≥110 ng/ml with a positive response to *P. citrinum* on LST or DIT as potential HP patients. Previous reports have investigated the criteria by which HP patients should be diagnosed. Tsushima et al. [2005] reported that serum KL-6 and SP-D may be related to the resolution of HP in addition to the LST values and chest CT. Kohno et al. [1989] and Kobayashi and Kitamura [1995] reported that the serum KL-6 is a good marker for disease activity in interstitial pneumonitis such as HP. SP-A and SP-D are also known to be good markers indicating disease activity of HP [Abe and Takahashi, 2000; Tanaka et al., 2000]. LST is sometimes used to screen patients and to find the causative antigen in some types of HP such as MWL [Tsushima et al., 2001]. In this study, KL-6, SP-D, and LST values in sera were significantly higher in the HP group than those in the non-HP group.

An ROC analysis was conducted to investigate the usefulness of these biomarkers as predictors. The AUC was the best of 0.99 for KL-6 when the cut-off level was determined to be 450 U/ml, and it was thought that KL-6 was the most useful as a predictor of HP in this study and that the cut-off level was appropriate. The AUC in LST was 0.87 when the cut-off level was determined to be 400%. This result suggested that the cut-off level of 400% might be appropriate. However, the positive rate in LST to *P. citrinum* was very high (45.8%) (Table II), and LST had low

TABLE III. Further Examinations: 9 Selected Workers and the Initial Patient

Case no.	Age, year/sex	Smoking history	Duration of working, year	Job	Symptoms	CXR	CT
(A) non-HP (n = 5)							
2	51/F	Never	0.25	Picker	Dry cough	Normal	Normal
3	54/M	Never	22	All	SOB	Normal	Normal
4	62/F	Never	37	Packer	(−)	Normal	Normal
5	51/M	Ex	21	All	(−)	Normal	Normal
7	72/F	Never	40	Packer	(−)	Normal	Normal
(B) HP (n = 5)							
1	54/F	Never	20	Picker/packer	Dry cough, SOB	GGO	GGO, CLSN
6	43/F	Never	3	Picker/packer	Dry cough	Normal	GGO
8	72/F	Never	16	Packer	Fever, SOB	GGO	GGO, CLSN
9	37/M	Ex	17	All	SOB	GGO	GGO, CLSN
Patient	47/F	Never	21	Picker/packer	Dry cough, SOB	GGO	GGO, CLSN

SOB, shortness of breath; GGO, gland-glass opacity; CLSN, centrilobular small nodule.

TABLE IV. BALF and TBLB Findings: 9 Selected Workers and the Initial Patient

Case no.	Cell counts, 10^4 cells/ml	Lymphocytes (%)	CD4/CD8	TBLB	Culture *Penicillium* spp. of BALF
(A) non-HP (n = 5)					
2	37.0	24.2	0.6	Normal	–
3	39.5	68.3	0.6	Normal	–
4	36.2	13.2	2.5	Normal	–
5	38.8	25.6	0.7	Normal	–
7	25.4	64.5	2.4	Normal	–
(B) HP (n = 5)					
1	45.8	91.5	0.36	Alveolitis	–
6	309.0	81	0.3	Alveolitis	+
8	11.9	61.8	1.9	Alveolitis	–
9	62.3	84.4	2.0	Alveolitis, Masson body	–
Patient	71.4	41.6	0.8	Alveolitis	–

BALF, bronchoalvelar lavage fluid; TBLB, transbronchial lung biopsy; CD, cluster of differentiation.

specificity. It is possible that the cut-off level should have been much higher. The level of inter-correlation among the biomarkers was the highest between KL-6 and SP-D (r = 0.90). KL-6 was independent of LST, and the measurement of both markers was useful to screen the HP patients in this study. The LST values in BALF were also significantly higher in the HP group (n = 5) than those in the Non-HP group (n = 5). It is possible that LST using BALF was useful to detect the causative antigen. In this study, Enoki and the culture medium were not identified as causative antigens. Interestingly, several participants had high LST values to even Enoki and the culture medium in the same way as *P. citrinum*. The cut-off levels in Enoki and the culture medium were both decided to be 400% because the lowest LST value to each antigen of normal controls was less than 400%.

DIT is also considered to be useful to screen HP patients to identify the cause of HP [Sanderson et al., 1992]. Precipitating lines were observed to *P. citrinum* in all of the HP patients. On the other hand, the line was also observed to *P. citrinum* in one of the 44 non-HP workers, who was working in a different farm where the HP patients worked. The sensitivity and specificity in DIT were both good at 100% and 97.5%, respectively, and DIT was helpful to screen HP patients and find the causative antigen in this study. However, some studies have reported that serum precipitating antibodies provided a useful marker of exposure to antigens but were not specific to an allergic respiratory

TABLE V. LST in Serum and BALF

	Enoki		Culture medium		*Penicillium citrinum*		
Case no.	Serum (%)	BALF (%)	Serum (%)	BALF (%)	Serum (%)	BALF (%)	Serum precipitins to *Penicillium citrinum*
(A) non-HP (n = 5)							
2	933	91	392	ND	457	112	–
3	572	110	712	ND	584	133	–
4	920	ND	396	ND	677	122	–
5	1,121	164	758	ND	859	582	–
7	1,932	71	1,116	ND	561	66	–
(B) HP (n = 5)							
1	111	240	544	768	1,661	3,518	+
6	263	353	342	ND	967	3,246	+
8	451	84	422	ND	1,696	321	+
9	118	134	263	204	281	1,096	+
Patient	147	300	237	210	715	5,679	+

LST, lymphocyte stimulation test; BALF, bronchoalvelar lavage fluid; ND, not done.
LST value ≥400% is considered to be positive on each antigen.

TABLE VI. The Comparison Among 48 Workers and the First HP Patient

	(A) non-HP (n = 44)	(B) HP (n = 5)	*P*-value (A) versus (B)	Cut-off level	AUC	Se. (%)	Sp. (%)
KL-6 (U/ml)	265.8 ± 136.2	2,790.4 ± 2,607.6	0.0004	450	0.99	100	93.2
SP-A (ng/ml)	31.8 ± 12.5	52.0 ± 33.0	0.0954	43.8	0.73	40	93.2
SP-D (ng/ml)	51.8 ± 29.7	239.6 ± 184.4	0.0037	110	0.90	60	93.2
LST (%)[a]	438.4 ± 547.0	1,064 ± 612.4	0.0105	400	0.87	80	56.8
DIT	1[b]	5[b]				100	97.5

AUC, area under the curve in ROC analysis; Se., sensitivity; Sp., specificity; DIT, double immunodiffusion test.
[a]LST to *Penicillium citrinum* in serum.
[b]Subjects with positive precipitins in sera to *Penicillium citrinum*.

disease such as HP [do Pico et al., 1976; Burrell and Rylander, 1981]. Tanaka et al. [2001] conducted a 3-year follow-up of allergy in workers cultivating Bunashimeji mushrooms, and reported that the positive rate of serum precipitins to the spores in non-HP workers was 30% in 1 year, 93% in 2 years, 94% in 3 years. These mushroom workers were exposed to Bunashimeji spores. It is possible that the difference in the positive rate in DIT occurred due to the exposure duration to antigens and the amount of antigens. Air sampling was not performed by month to month or year to year in this study, so the level of exposure to *P. citrinum* spores that the worker received cannot be assessed in this study. However, it was possible that *P. citrinum* began to grow very rapidly and the Enoki workers were suddenly exposed to large amounts of *P. citrinum* spores.

Penicillium spp. was commonly found indoors, and it is popular common fungi in Enoki farms. Enoki workers might have been exposed to small amounts of *P. citrinum* spores, Enoki spores and the organic matter in the culture medium for many years which were insufficient to develop HP. The workers may have been sensitized by these antigens. In the Enoki farm where the initial HP patient worked, *P. citrinum* was isolated throughout the work environment in places such as an air-conditioner [Yoshikawa et al., 2006]. *Rhizopus stolonifer* and Enoki were also isolated in that environment, but at a very low level. The investigation was not performed at every farm, however 77% of the workers reported seeing "green" fungus more frequently than usual during the study period, and *P. citrinum* was isolated from 54.2% of the workers. These results suggested that most Enoki workers were being exposed to large amounts of *P. citrinum* spores during that period.

According to the Japan Meteorological Agency, it seemed that precipitation in eastern Nagano prefecture during this study period was more than usual. The study area, which is located in eastern Nagano prefecture, is surrounded by mountains on four sides and is very humid all year round. This humid environment is appropriate for mushroom production. The fact that the area had an abundance of precipitation during this study period suggested that the humidity had been higher than usual. High humidity might have promoted the growth of *P. citrinum*. Furthermore, many workers reported that they often found "green" fungus in their work environments after the type of culture medium was changed 3 months earlier. The change of the culture medium might also be connected with the marked growth of *P. citrinum*.

Interestingly, two of 4 new HP patients were working at the same mushroom farm as the initial HP patient was. In this "epidemic" farm, high levels of *P. citrinum* was isolated throughout the work environment, and "green" fungus was found very easily on the culture medium and Enoki mushrooms themselves. Nine workers including the initial HP patient were involved with cultivating Enoki, and all 8 of the workers participated in this survey. Cases 1, 2, 3, and 8 were picked up for further examinations, and finally cases 1 and 8 were regarded as having HP. The rate of disease in this "epidemic" farm was 3/9 (33.3%). It was possible that the amounts of *P. citrinum* spores in the "epidemic" farm were much higher than those in the other farms. However, the differences of the amounts of the spores among Enoki farms cannot be discussed because air sampling was not performed at every Enoki farm.

In conclusion, 4 new cases of HP were found among workers cultivating Enoki mushrooms in this study. KL-6, SP-D, LST, and DIT were useful for detecting HP patients, and KL-6 was considered to be most useful predictor of HP. DIT was useful not only as a predictor of HP but also as a detector of the causative antigen. It appears that unclean indoor environments associated with changes in the weather and the culture medium promoted the rapid growth of *P. citrinum* and this stimulated the outbreak of HP.

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研究成果の刊行に関する一覧表
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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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永井英明	[今日の結核症－的確な診断から治療へ] 結核症の治療 合併症等を有する特殊な症例の治療	診断と治療	95	1973-1980	2007

Diagnosis of Active Tuberculous Serositis by Antigen-Specific Interferon-γ Response of Cavity Fluid Cells

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Background. To develop a more accurate methodology for diagnosing active tuberculous pleurisy, as well as peritonitis and pericardits of tuberculous origin, we established an antigen-specific interferon γ (IFN-γ)–based assay that uses cavity fluid specimens.

Methods. Over a 19-month period, 155 consecutive, nonselected patients with any cavity effusion were evaluated. Study subjects were 28 patients with bacteriologically confirmed active tuberculous serositis and 47 patients with definitive nontuberculous etiology. Culture was performed for 18 h with fluid mononuclear cells in the supernatant of the effusion together with saline or *Mycobacterium tuberculosis*–specific antigenic peptides, early secretory antigenic target 6 and culture filtrate protein 10. IFN-γ concentrations in the culture supernatants were measured.

Results. In patients with active tuberculous serositis, antigen-specific IFN-γ responses of cavity fluid samples were significantly higher than those of nontuberculous effusion samples. Area under the receiver operating characteristic (AUROC) curve was significantly greater for cavity fluid IFN-γ response (AUROC curve, 0.996) than for cavity fluid adenosine deaminase and whole-blood IFN-γ responses (AUROC curve, 0.882 and 0.719, respectively; $P = .037$ and $P < .001$, respectively). Although the AUROC curve was greater for cavity fluid IFN-γ response than for background cavity fluid IFN-γ level (AUROC curve, 0.975), the AUROC curves were not statistically significantly different ($P = .74$). However, multivariate logistic regression analysis revealed that cavity fluid IFN-γ responses were significantly associated with the diagnosis, even after adjustment for background IFN-γ level (adjusted odds ratio, 1.21; 95% confidence interval, 1.03–1.42; $P < .001$).

Conclusions. The cavity fluid IFN-γ assay could be a method for accurately and promptly diagnosing active tuberculous serositis.

Tuberculosis is a serious infectious disease that threatens the health of mankind, even in the modern world [1]. Active tuberculous pleuritis, peritonitis, and pericarditis should always be considered as possible causes of cavity fluid in the daily practice of clinical medicine. Their definitive diagnosis is based on the identification of *Mycobacterium tuberculosis* after culture of effusion or tissue biopsy specimens. However, the rate of positive results of such cultures of pleural fluid specimens is not high (<30%) [2]. In addition, because bacteriological results cannot be obtained rapidly, antituberculous agents should, in some cases, be instituted on the basis of clinical speculation without waiting for the definitive diagnosis. Measurement of nonspecific markers, such as adenosine deaminase (ADA) and IFN-γ, in the supernatant of fluid specimens has been used to diagnose pleural tuberculosis [3, 4]. A meta-analysis concluded that maximum joint sensitivity and specificity was 93% for the ADA assay and 96% for the IFN-γ assay [5]. It has been reported that the levels of these markers are also increased in the context of other diseases [6–9], and there is some contention as to the use of these tests [10, 11]. Thus, it would be desirable to develop a more specific diagnostic method for active tuberculous serositis (ATBS).

Active tuberculous pleuritis is thought to be caused by the spread of a small subpleural or hilar lymph node

Received 1 May 2007; accepted 2 August 2007; electronically published 8 November 2007.

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Clinical Infectious Diseases 2007; 45:1559–67

1058-4838/2007/4512-0005$15.00
DOI: 10.1086/523591

caseous focus of *M. tuberculosis* into the cavity, and T cells that specifically respond to tuberculous antigens and produce IFN-γ are recruited in the pleural cavity of patients with active tuberculous pleuritis [12, 13]. *M. tuberculosis*–specific protein antigens early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), which are absent from the genomes of all bacille Calmette-Guérin substrains and most nontuberculous mycobacteria, have been identified [14]. A specific method for the detection of tuberculous infection has been developed in which IFN-γ production (the number of IFN-γ–producing cells) is measured after stimulation of peripheral blood with these antigens in vitro [15–19]. We hypothesized that, when cavity fluid mononuclear cells of patients with ATBS were stimulated with the antigenic peptides ESAT-6 or CFP-10, IFN-γ responses that are greater than those achieved using the stimulation of peripheral blood might be expected. We report our results, which suggest the possibility of accurate diagnostic procedures for ATBS that reflect the local immune response in the cavity.

PATIENTS AND METHODS

Patients and evaluation of their cavity effusion specimens. Over a 19-month period from November 2004 through June 2006, 155 consecutive, nonselected patients with any cavity effusion were evaluated at the inpatient department of Tokyo National Hospital (Tokyo, Japan). Either cavity centesis or endoscopic procedures, including thoracoscopic and perioteneoscopic procedures, was performed for all patients; 75 patients for whom the etiology of cavity effusion could be definitively identified were enrolled. Table 1 shows the profiles and the clinical diagnoses that these patients received. All patients with tuberculosis had received <7 days of antituberculous chemotherapy at the time of testing. None of these patients had conditions, such as HIV infection, associated with severe immunocompromise, and none were receiving immunosuppressive drugs.

The differential diagnosis based on the evaluation of the pleural effusion specimens was made using the following criteria. A definitive diagnosis of active tuberculous pleuritis, peritonitis, or pericarditis was made on the basis of positive culture results and identification of *M. tuberculosis* in cavity effusion or parietal tissue specimens. With regard to nontuberculous pleuritis, peritonitis, and pericarditis, the diagnosis of neoplastic pleuritis, peritonitis, or pericarditis was based on detection of malignant cells in pleural effusion or tissue specimens; parapneumonic effusion was defined by the presence of fever, spu-

Table 1. Profiles of patients with cavity effusion.

Variable	Patients with active tuberculous serositis (n = 28)	Patients with nontuberculous effusion (n = 47)	All (n = 75)
Age, years			
Mean ± SD	60.5 ± 22	72.1 ± 10.8	67.9 ± 17.1
Range	20–91	46–91	20–91
Sex			
Male	24 (86)	40 (85)	65 (87)
Female	4 (14)	7 (15)	10 (13)
Clinical diagnosis			
Active tuberculous pleuritis	26	0	...
Active tuberculous peritonitis	1	0	...
Active tuberculous pericarditis	1	0	...
Nontuberculous pleuritis	0	45	...
Neoplastic pleuritis	0	24	..
Lung cancer	0	22	...
Malignant mesothelioma	0	2	...
Parapneumonic effusion	0	12	...
Heart failure	0	5	...
Asbestos pleural effusion	0	1	...
Posttraumatic pleural effusion	0	1	..
Mycobacterium avium pleuritis	0	1	...
Hypothyroidism (pleural effusion)	0	1	...
Cancerous peritonitis	0	1	...
Cancerous pericarditis	0	1	...

NOTE. Data are no. or no. (%) of patients, unless otherwise indicated.

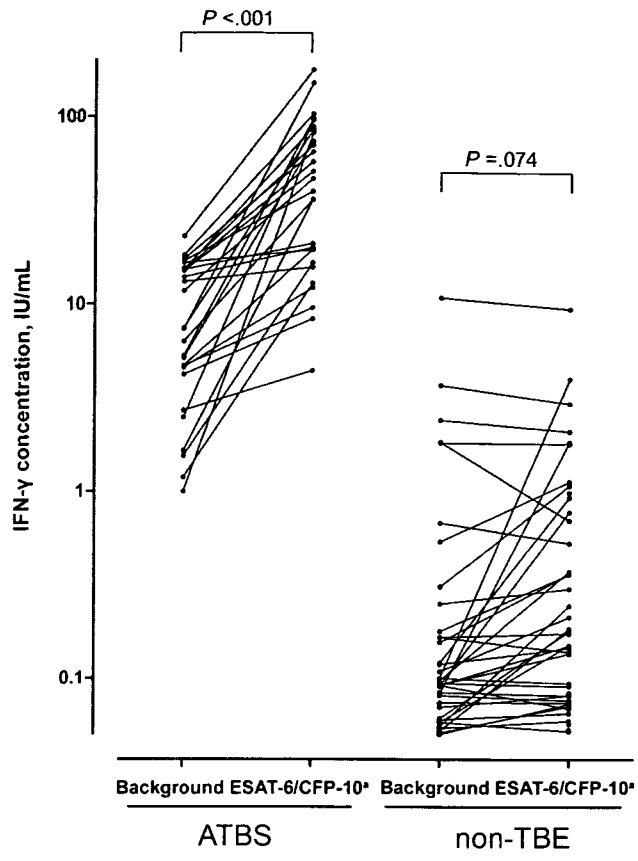

Figure 1. IFN-γ production in response to stimulation with *Mycobacterium tuberculosis*–specific antigen. Pleural, peritoneal, and pericardial fluid cells obtained from patients with active tuberculous serositis (ATBS) or nontuberculous effusion (non-TBE) were harvested and suspended in the individual supernatant of the effusion at 1–5 million cells/mL, stimulated with saline or *M. tuberculosis*–specific antigens, and incubated for 18 h, after which IFN-γ concentrations in the culture supernatant were measured by ELISA. If, in an initial assay, a supernatant was found to contain >15 IU/mL of IFN-γ, the specimen was diluted with zero standard and reassayed. IFN-γ concentrations in each patient, which were expressed as background saline control IFN-γ concentrations and concentrations after stimulation with specific antigens (early secretory antigenic target 6 [ESAT-6] and culture filtrate protein 10 [CFP-10]), are shown as closed circles connected with lines. Student's *t* test was used for differences between the ATBS and non-TBE groups. IFN-γ values after antigen stimulation are the measured values, not values subtracted from the corresponding background control values. [a]The higher IFN-γ concentrations after stimulation with either antigenic peptides ESAT-6 or CFP-10 are represented.

tum, infiltrates on chest radiograph, an infectious inflammatory response detected by serological testing, and improvement after the administration of antibiotics. Heart failure was assigned as the cause of pleural effusion if cardiac dysfunction was evident on ultrasonographic cardiograph and improvement was noted after the administration of diuretics or cardiotonic drugs. Asbestos pleural effusion was diagnosed on the basis of detection of asbestos particles or fibrous patches in pleural biopsy specimens. Posttraumatic hemothorax was indicated when there was evident pooling of blood in the thorax after chest trauma. Pleuritis due to *Mycobacterium avium* was considered on the basis of identification of *M. avium* in pleural effusion specimens.

Stimulation of cavity fluid cells with* M. tuberculosis*–specific antigens (cavity fluid IFN-γ assay). Twenty to 50 mL of cavity effusion were centrifuged at 2000 rpm for 15 min. The supernatant was separated, and the number of nucleated cells in the cell compartment was determined. Cells were then suspended at 1–5 million cells/mL in the individual supernatant of the effusion. One milliliter of this cell suspension was then added to 4 wells of 24-well plates (NUNC) and underwent culture in the presence of *M. tuberculosis* antigen peptides ESAT-6 or CFP-10 at a final concentration of 1 μg/mL (QuantiFERON-TB 2G; Cellestis) [19]. To the remaining 2 wells, physiological saline was added instead of the antigens, because a measurement of background IFN-γ of cavity fluid and phytohemagglutinin (5 μg/mL) was used as a positive control. Cells were incubated at 37°C for 18 h in a 5% CO_2 incubator, and the cultured supernatant was harvested for measurement of IFN-γ concentration. The value of the cavity fluid assay was defined as the difference between the determined higher value after stimulation with either ESAT-6 or CFP-10 and the value of the measurement of background IFN-γ. The whole-blood IFN-γ assay was performed as previously described [19].

Measurement of IFN-γ and ADA concentrations. IFN-γ concentration was measured using the QuantiFERON-CMI ELISA (Cellestis) according to procedures described in the manufacturer's instructions. The IFN-γ values were expediently replaced with 0.05 when the difference between the antigen-stimulated level and the background level was negative or <0.05 IU/mL (lower limit of measurement). If, in an initial assay, the supernatant was found to contain >15 IU/mL of IFN-γ (upper limit of measurement), the specimen was diluted with zero standard and reassayed. When both stimulated and background IFN-γ concentrations were extremely high, we performed another culture using plasma samples from each patient for medium to decrease the background IFN-γ concentration. The result of the whole-blood assay was considered to be indeterminate if the value for the positive control sample was <0.5 IU/mL after subtraction of the value for the negative control sample. ADA concentration in the supernatant of cavity fluid samples was measured by AUTO A MIZUHO ADA (Mizuho Medy). Three technicians with expertise in laboratory work were blinded to the clinical information of the patients, results of other assays in this study, and the reference standard.

Statistical analysis. Univariate analyses were performed using Student's *t* test and the Mann-Whitney *U* test. We constructed a receiver operating characteristic (ROC) curve by

Table 2. IFN-γ response in cavity fluid and whole-blood cells to tuberculous-specific and nonspecific antigens and adenosine deaminase (ADA) levels in patients with active tuberculous serositis and patients with nontuberculous effusion.

Variables	Patients with active tuberculous serositis (n = 28)	Patients with nontuberculous effusion (n = 47)	P
Background IFN-γ level in cavity fluid	7.4 (4.5–15.2)	0.09 (0.05–0.16)	<.001
ESAT-6–background[a]	26.4 (6.8–49.4)	0.05 (0.05–0.08)	<.001
CFP-10–background[b]	12.2 (2.8–37.7)	0.05 (0.05–0.09)	<.001
ESAT-6 or CFP-10–background[c]	34.8 (7.3–74.3)	0.05 (0.05–0.19)	<.001
Mitogen–background[d]	22.6 (2.1–55.1)	6.90 (0.32–21.5)	.009
Whole-blood IFN-γ assay result[e]	0.54 (0.3–0.9)	0.07 (0.05–0.54)	<.001
ADA concentration[f]	61.3 (43.0–71.5)	13.80 (7.95–27.5)	<.001

NOTE. Data are median value (interquartile range). Mann-Whitney U test was used for differences in IFN-γ and ADA concentrations between the patients with active tuberculous serositis and patients with nontuberculous effusion. CFP-10, culture filtrate protein 10; ESAT-6, early secretory antigenic target 6.

[a] Difference between the determined IFN-γ concentration after stimulation with ESAT-6 and the background IFN-γ concentration in cavity fluid.

[b] Difference between the determined IFN-γ concentration after stimulation with CFP-10 and the background IFN-γ concentration in cavity fluid.

[c] Difference between the determined higher IFN-γ concentration after stimulation with either ESAT-6 or CFP-10 and the background IFN-γ concentration.

[d] Difference between the determined IFN-γ concentration after stimulation with mitogen and the background IFN-γ concentration in cavity fluid.

[e] None of the patients had indeterminate results, and 1 patient with tuberculosis was unavailable.

[f] Not performed for 1 patient with tuberculosis.

plotting the rate of sensitivity against the rate of false-positive results over a range of cutoff values of IFN-γ and ADA for the assessment of diagnostic accuracy. Area under the ROC curve was calculated using the trapezoidal rule. Standard errors of the areas and comparisons between assays were calculated using a method described elsewhere [20]. Pairwise comparisons were made between area under the ROC curve for the cavity fluid IFN-γ assay and that for each of the following measures: background IFN-γ and ADA assays and the whole-blood IFN-γ assay. Adjustment for multiple comparisons was made using the Dunn-Sidák method. To assess whether the cavity fluid assay lends additional diagnostic information to that provided by the background IFN-γ level, these 2 measurements were introduced as predictors in a multivariate logistic regression model. These 2 explanatory variables were treated as continuous variables. $P < .05$ was considered to be statistically significant. All statistical analyses were performed using Stata, version 9 (Stata).

This study was approved by the ethics review committee of our hospital. We obtained informed consent from all participants in the study.

RESULTS

The mean age of patients with nontuberculous effusion (non-TBE) was significantly higher than that of patients with ATBS (72.4 years vs. 60.5 years; $P = .013$). There was no statistically significant difference in sex between the groups (85% male vs. 81% male; $P = .745$) (table 1).

Figure 1 shows results of the cavity fluid IFN-γ assay for each subject (in both the ATBS and non-TBE groups), including background IFN-γ values and the highest values for either of the stimulation antigens, ESAT-6 or CFP-10. The IFN-γ concentration was significantly increased after antigen stimulation, compared with the background IFN-γ concentration, in every patient with ATBS ($P < .001$). However, in patients with non-TBE, IFN-γ concentrations did not significantly increase after stimulation with antigens ($P = .074$)

The median values and the interquartile range of background cavity fluid IFN-γ, antigen-specific cavity fluid IFN-γ, whole-blood, and ADA assays are shown in table 2. Individual values for these parameters, as well as the result of the cavity fluid assay multiplied by the background IFN-γ level, are indicated by dots in figure 2. Background IFN-γ concentrations were significantly higher in patients with ATBS than in patients with non-TBE. Cavity fluid IFN-γ responses to both ESAT-6 and CFP-10 were significantly greater for patients with ATBS than for patients with non-TBE. Likewise, when an IFN-γ response was represented by a higher value of response to either ESAT-6 or CFP-10, the difference between the values for the ATBS group and the non-TBE group was greater. IFN-γ responses to mitogen were significantly greater for patients with ATBS than

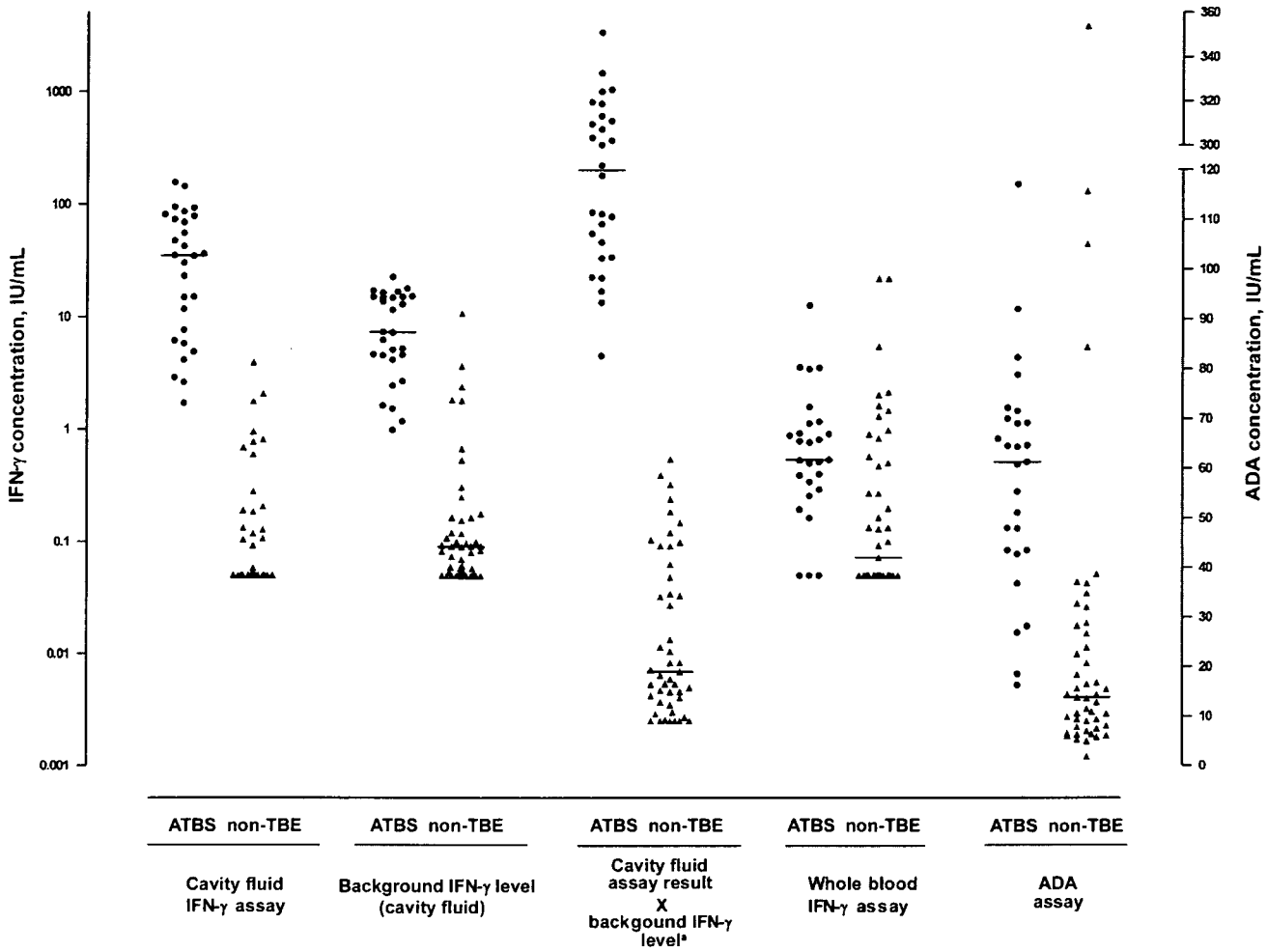

Figure 2. IFN-γ concentrations in cavity fluid and whole-blood and adenosine deaminase (ADA) concentrations in patients with active tuberculous serositis (ATBS) and in patients with nontuberculous effusion (non-TBE). Individual results of whole-blood IFN-γ, ADA, cavity fluid IFN-γ, and background control IFN-γ assays for patients with ATBS or non-TBE are shown. IFN-γ values are represented by the higher value of response to either early secretory antigenic target 6 (ESAT-6) or culture filtrate protein 10 (CFP-10) after subtraction of each background control IFN-γ value for the cavity fluid and whole-blood IFN-γ assays. Values for patients with ATBS and non-TBE are represented by closed circles and triangles, respectively. Horizontal lines in the columns represent the median value in each group. [a]Values calculated by multiplying the result of the cavity fluid IFN-γ assay by the background cavity fluid IFN-γ level and expressed as IU/mL^2.

for those with non-TBE. The median value of IFN-γ in the whole-blood IFN-γ assay was significantly higher for patients with ATBS than for patients with non-TBE, as was the median ADA value. For the whole-blood assay, none of the patients had indeterminate results, and 1 patient with ATBS was unavailable for testing. The ADA assay was not performed for 1 patient with tuberculosis.

Relative discriminative accuracy of the whole-blood, ADA, background IFN-γ, and cavity fluid assays was assessed in the area under the ROC curve (figure 3, table 3). The relative discriminative accuracy of the cavity fluid assay was statistically significantly different from that of the ADA ($P = .037$) and whole-blood assays ($P < .001$). The ROC curve for the cavity fluid assay was bowed further to the upper left, compared with that for the background IFN-γ level, but the areas under the ROC curve for the 2 assays were not statistically significantly different at the 5% level of type I error rate ($P = .74$).

Multivariate logistic regression was used as an aid in assessment of whether the cavity fluid assay added any diagnostic information to that yielded by the background IFN-γ level (table 4). Spearman's coefficient of correlation between the 2 measurements was 85%. The 2 measurements did not display statistical evidence of an interaction ($P = .84$). The Hosmer-Lemeshow test did not indicate a lack of fit ($P = .74$); thus, the predictors provide for a reasonably well calibrated model without data transformation. The cavity fluid assay displayed

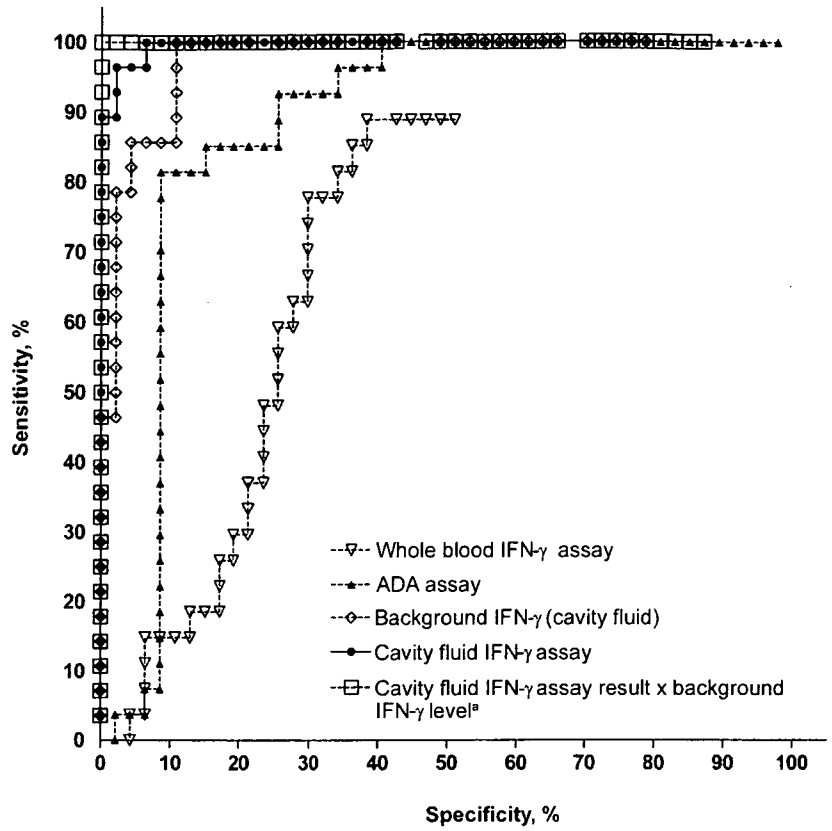

Figure 3. Receiver operating characteristic curves for 5 diagnostic methods used for patients with active tuberculous serositis and nontuberculous effusion. [a]Values calculated by multiplying the result of the cavity fluid IFN-γ assay by the background cavity fluid IFN-γ level and expressed as IU/mL[2].

a statistically significantly nonzero level of association with diagnostic status, even after adjustment for background IFN-γ concentrations, which supports the contention that the cavity fluid assay provides for improved diagnostic accuracy over that provided by the background IFN-γ level alone.

The cutoff value for each test was chosen to maximize specificity without significant loss of sensitivity. Table 5 shows sensitivity, specificity, likelihood ratio, and predictive value by cross classification. To calculate the predictive value, ~3.2% of pretest probability of ATBS in our specialist hospital for tuberculosis was used. As a result, both the likelihood ratio and predictive value of the cavity fluid assay were greater than those of the other assays. Furthermore, the value from when the results of the cavity fluid assay were multiplied by the background IFN-γ level was the most sensitive and specific predictor of ATBS.

DISCUSSION

Area under the ROC curve is the primary index for assessing the discriminative accuracy of a diagnostic method. Using this index, the cavity fluid assay displayed greater ability to discriminate ATBS than did either the ADA or whole-blood assays. The ROC curve index was not able to detect a difference in discriminative accuracy between unstimulated and specific antigen-stimulated IFN-γ response ex vivo. Nevertheless, a diagnostic advantage in assaying specific antigen-stimulated IFN-γ response was evident such that a multivariate logistic regression model provides a better fit to the clinical diagnosis when specific antigen-simulated IFN-γ production is included; the regression coefficient for the cavity fluid IFN-γ assay is statistically significantly different from zero. This better fit indicates improved calibration of the model. It is recognized that

Table 3. Comparison of diagnostic accuracy by area under the receiver operating characteristic (AUROC) curves.

Variable	AUROC curve (95% CI)	SE
Whole-blood IFN-γ assay	0.719 (0.598–0.838)	6.1
ADA assay	0.882 (0.799–0.965)	4.2
Background IFN-γ of cavity fluid	0.975 (0.946–1.004)	1.5
Cavity fluid IFN-γ assay[a]	0.996 (0.989–1.004)	0.4

[a] The value of the cavity fluid IFN-γ assay was defined as the difference between the determined higher IFN-γ value after stimulation with either early secretory antigenic target 6 or culture filtrate protein 10 and the background IFN-γ concentration.

Table 4. Multivariate logistic regression analysis.

Variable	Regression coefficient, log odds per IU/mL	SE	Likelihood ratio[a]	*P*	OR (95% CI)
Intercept	−3.47	0.79	...	...	...
Background IFN-γ of cavity fluid	0.34	0.23	3.10	.08	1.40 (0.80–2.19)
Cavity fluid IFN-γ assay	0.19	0.08	18.98	<.005	1.21 (1.03–1.42)

NOTE. Two explanatory variables were treated as continuous variables. Standardized ORs (each variable scaled to its interquartile range) were 5.6 for background IFN-γ and 55 for cavity fluid IFN-γ assay. Spearman's coefficient of correlation between the 2 measurements was 85%. The 2 measurements did not display statistical evidence of an interaction ($P = .84$). The Hosmer-Lemeshow test did not indicate a lack of fit ($P = .74$); thus, the predictors provide for a reasonably well calibrated model without data transformation.

[a] Likelihood ratio was determined using χ^2 test statistics and was single-degree-of-freedom.

area under the ROC curve is important but not always optimum in assessment of diagnostic tests, especially for predictive assessment of risk [21].

For the IFN-γ assay using cavity fluid, the IFN-γ concentration was significantly increased after antigen stimulation, compared with the background IFN-γ concentration, for every patient with ATBS (figure 1). It may be speculated that this result was because of further IFN-γ production by antigen-specific T cells in response to stimulation. We have obtained similar results for 30 patients with pleural effusion in whom active tuberculous pleuritis was strongly suspected clinically. Although, in these patients, definitive bacteriological evidence could not be obtained from pleural effusion specimens, antituberculous chemotherapy was effective (data not shown). In contrast, for the majority of 47 patients with effusion of nontuberculous etiology, IFN-γ concentrations did not increase after stimulation with antigens. This was also true for 5 of 47 patients with nontuberculous pleuritis who had relatively high background IFN-γ concentrations (>1 IU/mL).

In 3 of the patients with non-TBE who had a history of prior tuberculosis and in some of those without any clinical history of tuberculosis, IFN-γ concentrations were slightly increased in cavity fluid after stimulation, compared with background IFN-γ concentrations. Similarly, results of the whole-blood assay were positive for all of these patients. It may be speculated that when patients with a history of tuberculosis encounter serositis attributable to causes other than tuberculosis, preexisting antigen-specific memory T cells in peripheral blood also appear in the effusion and produce IFN-γ through stimulation by antigens in vitro. If diagnostic evaluation was made only on the basis of measuring an antigen-specific IFN-γ response or on a count of IFN-γ–producing cells by enzyme-linked immunospot assay [22], there is a possibility that some patients with an effusion of nontuberculous etiology who have incidental latent tuberculosis infection (LTBI) will receive a false-positive diagnosis. In such cases, non-TBE with LTBI can be differentially diagnosed from ATBS on the basis of low background IFN-γ concentration (figure 1). We speculate that high background IFN-γ concentrations in patients with ATBS are a reflection of the ongoing active status of the local type I helper T cellular immune response. High IFN-γ concentrations in the supernatant of pleural effusion specimens from patients with tuberculous pleuritis have been reported [3, 7, 23, 24]. However, it has also been demonstrated that the IFN-γ concentration can be high in those patients with an effusion of nontuberculous etiology [7]. In our study, 5 of 47 patients with non-TBE (i.e., *M. avium* pleuritis, cancerous pleuritis, and parapneumonia) had background IFN-γ concentrations that were similar to those found in patients with ATBS (>1 IU/mL). Thus, it appears that specificity of the background IFN-γ level may be limited and that false-positive responses can occur. The cavity fluid assay can assess both background IFN-γ and antigen-specific IFN-γ responses simultaneously, and it is possible to compensate for the fault mutually by assessing the value of both responses together. We demonstrated that the values from when the result of the cavity fluid assay was multiplied by the result of the background IFN-γ possibly could be used to accurately diagnose active tuberculous effusion (figure 2, table 5).

Although both the enzyme-linked immunospot assay using the peripheral blood and whole-blood IFN-γ assay have been reported for the diagnosis of tuberculosis, the results of these assays were also positive for people with LTBI. The whole-blood assay also has been reported to be highly specific for *M. tuberculosis* infection, but it cannot discriminate between active tuberculosis and LTBI. In our study, the group of patients who did not have ATBS likely contained a number of subjects with LTBI. Indeed, 3 patients with non-TBE who had a documented history of prior tuberculosis had positive whole-blood assay results. Similarly, 11 other patients who did not have ATBS had positive whole-blood assay results but had no clear history of tuberculosis or evidence of an old tuberculous lesion on chest radiograph (table 5). Because of the high specificity (>98%) of the whole-blood IFN-γ assay among a young population with no risk for tuberculosis [19], we speculated that these patients were latently infected with tuberculosis, reflecting their age and an era when tuberculosis was prevalent in Japan. An additional

Table 5. Comparison of diagnostic accuracy of adenosine deaminase (ADA), whole-blood, and cavity fluid IFN-γ assays.

Variable	Cutoff value	Result, positive: negative		Sensitivity, % (95% CI)	Specificity, % (95% CI)	Positive likelihood ratio	Negative likelihood ratio	PPV[a]	NPV[a]
		ATBS group	Non-TBE group						
Whole-blood IFN-γ assay	0.281	21:6	14:33	77.8 (57.7–91.4)	70.2 (55.1–82.7)	2.6	3.2	7.94	99.0
ADA assay	40.700	22:5	4:43	81.5 (61.9–93.7)	91.5 (79.6–97.6)	9.6	4.9	24.5	99.3
Background IFN-γ of cavity fluid	2.456	24:4	2:45	85.7 (67.3–96.0)	95.6 (85.5–99.5)	20.1	6.7	39.7	99.5
Cavity fluid IFN-γ assay[b]	2.352	27:1	1:46	96.4 (81.7–99.9)	97.8 (88.7–99.95)	45.3	27.4	60.3	99.9
Cavity fluid assay result times the background IFN-γ level[c]	2.590	28:0	0:47	100 (87.7–100)	100 (92.5–100)	...	...	100.0	100.0

NOTE. For the whole-blood assay, none of the patients had indeterminate results, and 1 patient with active tuberculous serositis (ATBS) was unavailable. The ADA assay was not performed for 1 patient with ATBS. Non-TBE, nontuberculous effusion; NPV, negative predictive value; PPV, positive predictive value.

[a] Pretest probability of active tuberculous effusion in our department of respiratory medicine was 3.2%

[b] The result of the cavity fluid IFN-γ assay was defined as the difference between the determined higher value of IFN-γ after stimulation with either early secretory antigenic target 6 or culture filtrate protein 10 and the background IFN-γ concentration.

[c] The values from when the result of the cavity fluid IFN-γ assay was multiplied by the background IFN-γ level.

finding was that the whole-blood assay result was negative for 6 of 27 patients with ATBS. In this study, results of the whole-blood assay showed reduced sensitivity (77.8%), compared with the sensitivity found in previous reports (89%) [19]. It has been reported that T cells that are specific for tuberculous antigens are sequestered from the circulation to the pleural cavity in patients with pleural tuberculosis [22]. The migration of antigen-specific T cells from peripheral blood to the active site of disease was thought to be 1 possible cause of lower than previously reported sensitivity of the whole-blood assay.

ADA in the supernatant of pleural effusion is reported as a diagnostic marker for tuberculous pleuritis [25–27]. The sensitivity and specificity of the ADA assay were reported to be 47.1%–100% and 0%–100%, respectively [28]. In our study, ADA concentrations were significantly higher in patients with ATBS than in patients with non-TBE. With regard to both sensitivity and specificity, the ADA assay had better performance than did the whole-blood assay for diagnosis of ATBS (table 5). However, in 4 of 47 patients with non-TBE (attributable to asbestosis, adenocarcinoma, and parapeumonic pleural effusion), ADA concentrations were as high as those in patients with ATBS. Furthermore, in some patients with active pleural and peritoneal tuberculosis, the ADA concentration was as low as that in patients with non-TBE. These false-positive and false-negative cases will be problematic when physicians make decisions regarding the initiation of long-term antituberculous chemotherapy.

In conclusion, we reported a highly sensitive and specific diagnostic method for active tuberculous pleuritis, tuberculous peritonitis, and pericarditis in which IFN-γ responses were measured after stimulation of cavity fluid cells with *M. tuberculosis*–specific antigens. The cavity fluid IFN-γ assay could be a noninvasive method for accurately and promptly diagnosing tuberculous serositis in patients in whom active tuberculosis in the cavity space is clinically suspected but for which no bacteriological evidence can be obtained.

Acknowledgments

We thank Yurika Ito, Ayako Watanabe, and Yasuko Inoue, for their technical assistance with the laboratory assays, and Drs. Kazuhiro Uchiyama, Toshiki Tamura, and Hajime Goto, for their support.

Financial support. The Ministry of Health, Labor and Welfare of Japan.

Potential conflicts of interest. All authors: no conflicts.

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