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Chemokine (C-C motif) ligand 3 detection in the serum of persons exposed to asbestos: A patient-based study

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Key words

Asbestos, biological markers, chemokine CCL3, Environmental carcinogens, mesothelioma

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Exposure to asbestos results in serious risk of developing lung and mesothelial diseases. Currently, there are no biomarkers that can be used to diagnose asbestos exposure. The purpose of the present study was to determine whether the levels or detection rate of chemokine (C-C motif) ligand 3 (CCL3) in the serum are elevated in persons exposed to asbestos. The primary study group consisted of 76 healthy subjects not exposed to asbestos and 172 healthy subjects possibly exposed to asbestos. The secondary study group consisted of 535 subjects possibly exposed to asbestos and diagnosed with pleural plaque (412), benign hydrothorax (10), asbestosis (86), lung cancer (17), and malignant mesothelioma (10). All study subjects who were possibly exposed to asbestos had a certificate of asbestos exposure issued by the Japanese Ministry of Health, Labour and Welfare. For the primary study group, levels of serum CCL3 did not differ between the two groups. However, the detection rate of CCL3 in the serum of healthy subjects possibly exposed to asbestos (30.2%) was significantly higher ($P < 0.001$) than for the control group (6.6%). The pleural plaque, benign hydrothorax, asbestosis, and lung cancer groups had serum CCL3 levels and detection rates similar to that of healthy subjects possibly exposed to asbestos. The CCL3 chemokine was detected in the serum of 9 of the 10 patients diagnosed with malignant mesothelioma. Three of the patients with malignant mesothelioma had exceptionally high CCL3 levels. Malignant mesothelioma cells from four biopsy cases and an autopsy case were positive for CCL3, possibly identifying the source of the CCL3 in the three malignant mesothelioma patients with exceptionally high serum CCL3 levels. In conclusion, a significantly higher percentage of healthy persons possibly exposed to asbestos had detectable levels of serum CCL3 compared to healthy unexposed control subjects.

Inhalation of asbestos elicits a high risk of developing lung and mesothelial diseases, including fatal malignant mesothelioma. Although the production and use of asbestos is now limited in many countries, asbestos is still widely used.[1] In addition, due to the long latency period of asbestos-associated disease development, even in countries that have restricted the use of asbestos, past exposure remains a serious public health issue. The mortality due to malignant mesothelioma alone in the USA, Europe, Japan, and Australia, regions with strong health controls in place, is predicted to be more than 400 000 between the years 2005 and 2045,[2] and the yearly worldwide mortality due to all asbestos exposure-related diseases is predicted to be 100 000–140 000.[3] Careful follow-up of patients exposed to asbestos is a key issue in controlling the development of asbestos-associated diseases. Accordingly, identification of healthy asymptomatic persons exposed to asbestos is an

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important goal. Testing for asbestos exposure is particularly relevant for persons who work or previously worked in asbestos factories, residents who lived near asbestos factories, workers processing rubble resulting from destruction of asbestos-containing homes and buildings, and firefighters and other rescue workers.

Numerous studies searching for biomarkers of asbestos exposure and malignant mesothelioma, with the majority concentrating on malignant mesothelioma, have been carried out, and a number of markers have been proposed.[4–32] Most of these studies, however, suffer from small patient numbers, and consequently, the diagnostic value of most proposed markers requires further evaluation. Osteopontin (OPN) and soluble mesothelin-related proteins (SMRP), as defined in Cristaudo *et al.* 2011,[33] have generally been regarded as the most promising biomarkers.[13,33–42] Application of OPN, however, is limited: OPN is not able to discriminate between asbestos-exposed subjects without malignant mesothelioma and unexposed subjects,[11,34] and OPN is not specific to mesothelioma.[34,43–48] Initially, SMRP was also found to be limited to detection of malignant mesothelioma,[4,34] however, a later study reported that SMRP might also serve as a marker of asbestos exposure.[10] These conflicting results remain to be resolved. Another promising biomarker is fibulin-3;[22] however, fibulin-3 cannot distinguish asbestos-exposed subjects without malignant mesothelioma from unexposed subjects.[22] Therefore, establishment of biomarkers that detect asbestos exposure, and consequently identify persons at risk of developing asbestos-associated diseases, including malignant mesothelioma, remains an important goal.

In rats treated with nanoscale titanium dioxide by intrapulmonary instillation, macrophages interact with TiO_2 aggregates in the lung and produce chemokine (C-C motif) ligand 3 (CCL3), also known as macrophage inflammatory protein 1-α, resulting in increased levels of CCL3 in the blood.[49] Based on this finding, we undertook the current patient-based study to determine whether the serum levels or the detection rate of CCL3 are elevated in asbestos-exposed subjects.

In this study, we determined the serum CCL3 levels in healthy asymptomatic subjects possibly exposed to asbestos and in healthy unexposed subjects. We also determined the serum CCL3 levels in patients possibly exposed to asbestos and diagnosed with pleural plaque, benign hydrothorax, asbestosis, lung cancer, and malignant mesothelioma. Our primary finding was that a significantly higher percentage of healthy asymptomatic persons possibly exposed to asbestos had detectable levels of serum CCL3 compared to healthy unexposed control subjects.

Materials and Methods

Ethics statement. This study was approved by the Ethics Review Committee of the respective participating institutes and hospitals: Nagoya City University Graduate School of Medical Sciences (Nagoya, Japan), Asahi Rosai Hospital (Owariasahi, Japan), Saiseikai Chuwa Hospital (Sakura, Japan), and Nagoyashi (Nagoya City) Koseiin Medical Welfare Center (Nagoya, Japan), and conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Tokyo 2004). Participants provided written informed consent before inclusion in the study, after which serum samples were obtained, coded, and stored in aliquots at −80°C until use.

Subjects. *Serum of unexposed subjects.* Control sera were collected from the teaching and research staff at the Nagoya City University Medical School and healthy inmate residents /patients at Nagoyashi Koseiin Medical Welfare Center Hospital (Koseiin Hospital) (n = 76; mean age, 50.9 ± 17.7 years). These subjects had no history of work or tenancy at asbestos-related workplaces or residences. They were free from lung and pleural lesions on periodical (once or twice a year) institutional health examinations including physical, chest x-ray, blood biochemical, and electrocardiogram examinations.

Serum of exposed subjects. The sera of subjects possibly exposed to asbestos (n = 707; mean age, 69.1 ± 8.2 years) were collected from patients who visited or were hospitalized in the Japan Labour Health and Welfare Organization Asahi Rosai (work-related accident) Hospital, the Saiseikai Chuwa Hospital, or the Nagoya City University Hospital from 2008 to 2012. All of the enrolled subjects potentially exposed to asbestos had certified documents issued by the Japanese Ministry of Health, Labour and Welfare for the compensation of medical care. The exposed subjects were grouped as follows: no detectable lesions (n = 172), pleural plaque (including 12 cases of pneumoconiosis complication) (n = 412), benign hydrothorax (n = 10), asbestosis lung (asbestosis) (n = 86), lung cancer (n = 17), and malignant mesothelioma (n = 10). The diagnosis for all lung and mesothelial disease cases was made by chest x-ray and/or computed tomography examinations. The diagnosis of malignant tumors was made by endoscopic examination coupled with histopathological examination of biopsy specimens. Pathological examination of malignant mesothelioma included an immunohistochemical antibody panel; positive markers were calretinin, mesothelin, WT1 (Wilms tumor 1), D2-40 (mAb directed against M2A antigen), and CK5/6 (cytokeratin 5/6). For malignant mesothelioma diagnosis, staining with at least two positive markers must be positive and carcinoembryonic antigen must be negative. In addition, thyroid transcription factor 1 and Ber-EP4 staining should be negative (see also ref. 50). All the malignant mesothelioma cases were epithelial-type tumors. For all subjects, job history and the site of residence were recorded. Residents near asbestos factories without any history of asbestos-related occupation were certified as asbestos-exposed and included in the asbestos-exposed groups. For smokers, previous or current smoking status was recorded and expressed as smoking index (Brinkman index: daily number of cigarettes × years of smoking).

Enzyme-linked immunosorbent assay. Human serum CCL3 was measured using the Quantikine Human CCL3/MIP-1a Immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, except that the samples added to the ELISA plates were incubated at room temperature for 3 h instead of 2 h. Levels of CCL3 below the detection limit (7.8 pg/mL) were arbitrarily regarded as 0. The association of CCL3 levels with the subject's work place, work duties, length of exposure, lapse of time after the last exposure, and smoking habit was analyzed.

Immunohistochemistry of malignant mesothelioma. Four biopsy cases and one autopsy case with malignant mesothelioma were available and examined by immunohistochemistry for the presence of CCL3, C-ERC/mesothelin (mesothelin), and CD68, a macrophage marker. (The autopsy case and three of the four biopsy cases were available for analysis of serum CCL3.) Slides of malignant mesothelioma were deparaffinized and heated in 10 mM sodium citrate, 0.05% Tween 20 (pH 6.0) for 10 min for antigen retrieval. The slides were blocked with Blocking One (03953-95; Nacalai Tesque, Kyoto, Japan) and incubated with rabbit anti-human CCL3 polyclonal antibodies (LS-B1056; Lifespan Biosciences, Seattle, WA, USA)

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diluted 1:100 at 4°C overnight and then washed and incubated with Alexa Fluor 488 labeled anti-rabbit secondary antibodies diluted 1:500 (Invitrogen Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. The slides were then washed with Blocking One for 30 min and incubated with rabbit anti-human mesothelin mAbs (ab93620; Abcom, Tokyo, Japan) diluted 1:100 at 4°C overnight, and then washed and incubated with Alexa Fluor 546 labeled anti-rabbit secondary antibodies (Invitrogen Molecular Probes) diluted 1:500 for 1 h at room temperature.

Statistics. The Kruskal–Wallis test was used to analyze the levels of CCL3 in the serum. Spearman's rank correlation coefficient was used to analyze the associations of CCL3 level and background factors: age, gender, cigarette consumption (scored by the Brinkman index), the length of exposure time to asbestos, and the lapse of time after the last potential exposure to asbestos. The Steel–Dwass method was used to compare CCL3 levels among the asbestos-exposed subgroups: no lesion, pleural plaque, benign hydrothorax, asbestosis lung, lung cancer, and malignant mesothelioma. The effects of background factors on detection of serum CCL3 was analyzed using multivariable logistic regression, and CCL3 detection was analyzed using multivariable logistic regression adjusted by background factors. P-values < 0.05 were considered to indicate statistical significance. Statistical analyses were carried out using JMP version 9.0 (SAS Institute, Cary, NC, USA).

Results

Study population. The primary study population was composed of 76 healthy subjects not exposed to asbestos and 172 healthy, asymptomatic (i.e., no detectable lung or pleural lesions) patients possibly exposed to asbestos. The general characteristics of the primary study group are summarized in Table 1a.

The secondary study population was composed of 535 subjects possibly exposed to asbestos and diagnosed with pleural plaque (412), asbestosis (86), benign hydrothorax (10), lung cancer (17), and malignant mesothelioma (10). The general characteristics of the secondary study group are summarized in Table 1b.

Table 1. General characteristics of the (a) primary study group, consisting of healthy subjects exposed or not exposed to asbestos (b) secondary study group, composed of subjects possibly exposed to asbestos and diagnosed with lung disease

	Diagnosis	No.	Gender		Age, years
			Male	Female	
(a)					
Unexposed	No lesions	76	48	28	50.9 ± 17.7
Asbestos exposed	No lesions	172	141	31	65.7 ± 8.8
(b)					
Exposed to asbestos	Pleural plaque†	412	315	97	69.7 ± 7.8
	Asbestosis	86	67	19	71.6 ± 6.9
	Benign hydrothorax	10	9	1	70.5 ± 6.2
	Lung cancer	17	17	0	73.5 ± 7.4
	Malignant mesothelioma	10	9	1	69.9 ± 5.6

†Includes 12 cases of pleural plaque with pneumoconiosis (mainly silicosis).

All study participants, with the exception of the 76 healthy subjects not exposed to asbestos, had certificates of asbestos exposure issued by the Japanese Ministry of Health, Labour and Welfare. However, confirmation of the presence of asbestos fibers in the lung or pleural tissues of healthy, asymptomatic persons is not possible. Therefore, in the primary study group the study participants with certificates of asbestos exposure must be assumed to be possibly exposed to asbestos, resulting in this study group being composed of an above average number of persons exposed to asbestos rather than being composed entirely of asbestos-exposed persons. Consequently, these study subjects are referred to as healthy, asymptomatic subjects possibly exposed to asbestos in this report.

Serum CCL3 levels: Primary study group. The serum CCL3 levels in the unexposed group and the healthy, asymptomatic subjects possibly exposed to asbestos are shown in Figure 1. For the study participants with detectable serum CCL3, there was no difference in CCL3 levels between the healthy control subjects and the healthy, asymptomatic subjects possibly exposed to asbestos. The study data can be downloaded from Table S1.

Serum CCL3 levels and background factors: Primary study group. Age and cigarette consumption (scored by the Brinkman index) showed a significant association with serum CCL3 levels (Table 2). Gender, the length of exposure time to asbestos, and the lapse of time after the last potential exposure to asbestos did not show a significant association with serum CCL3 levels.

Detection of serum CCL3: Primary study group. Subjects with CCL3 levels higher than 7.8 pg/mL, the detection limit of the ELISA assay, were defined as positive for serum CCL3. The detection rate of CCL3 in the serum of the primary study group is shown in Table 3. The detection rate of serum CCL3 in healthy, asymptomatic subjects possibly exposed to asbestos (52/172; 30.2%) was significantly higher (see Table 4) than in the unexposed control group (5/76; 6.6%).

Detection of serum CCL3 and background factors: Primary study group. Age, gender, smoking habit (never, previous, or current smoker), cigarette consumption (scored by the Brinkman index), the length of exposure time to asbestos, and the lapse of time after the last exposure to asbestos did not show a significant association with detection of CCL3 in the serum.

Levels of CCL3: Secondary study group. The serum CCL3 levels in the secondary study population are shown, alongside the levels in the primary study population, in Figure 2. For the study participants with detectable serum CCL3 in the pleural plaque, asbestosis, benign hydrothorax, and lung cancer groups, CCL3 levels were not different between groups or from the healthy, asymptomatic subjects possibly exposed to asbestos. In contrast, detectable serum CCL3 levels in the 10 patients constituting the mesothelioma group were significantly higher compared to the other groups. Notably, the higher levels of serum CCL3 in the mesothelioma group was entirely due to the levels in three patients with extraordinarily high – 611, 1007, and 2012 pg/mL – serum CCL3 levels. The study data can be downloaded from Table S1.

Detection of serum CCL3: Secondary study group. The detection rate of CCL3 in the serum of the study subjects with pleural plaque (139/412; 33.7%), asbestosis (34/86; 39.5%), benign hydrothorax (3/10; 30.0%), and lung cancer (5/17; 29.4%) was similar to that of the healthy, asymptomatic subjects possibly exposed to asbestos (Table 5): there were no

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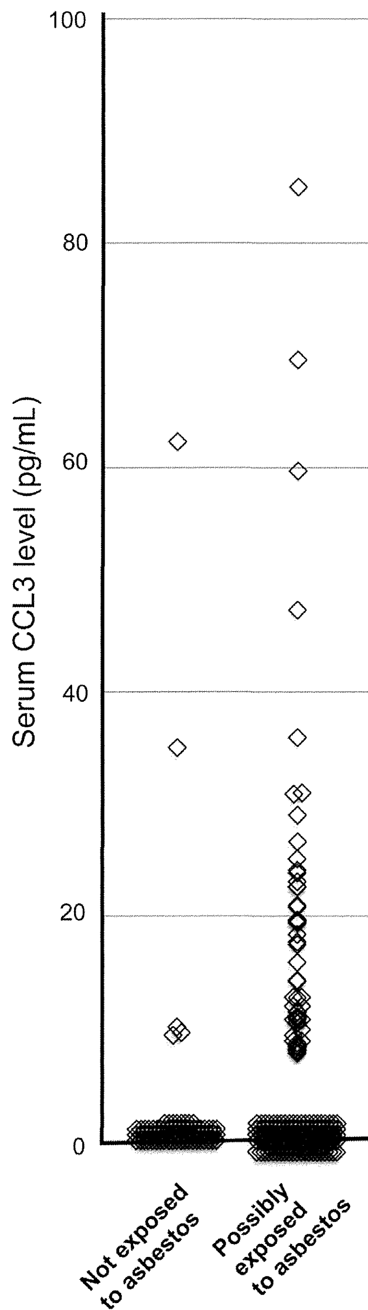

Fig. 1. Serum chemokine (C-C motif) ligand 3 (CCL3) levels in the primary study group. Levels of CCL3 in subjects not exposed to asbestos (controls) and in healthy, asymptomatic subjects possibly exposed to asbestos. Excluding the subjects without detectable CCL3, serum CCL3 levels in the group composed of healthy, asymptomatic subjects possibly exposed to asbestos are not different from the five control participants with detectable CCL3 in their serum.

Table 2. Associations between chemokine (C-C motif) ligand 3 levels with background factors

Background factor	Rho	*P*-value
Age	+0.196	0.002
Cigarette consumption	+0.171	0.026
Gender (M = 0; F = 1)	−0.052	0.417
Length of exposure time	+0.070	0.431
Lapse of time since last exposure	−0.080	0.359

F, female; M, male.

Table 3. Detection of serum chemokine (C-C motif) ligand 3 in the primary study group, consisting of healthy subjects exposed or not exposed to asbestos

Lesion category	Total number of subjects	Number of positive subjects	Detection rate, %	95% confidence interval
Unexposed				
No lesions	76	5	6.6	0.3–14.5
Asbestos exposed				
No lesions	172	52	30.2	23.9–37.5

Table 4. Odds ratio for asbestos exposure. The detection rate of serum CCL3 in healthy, asymptomatic subjects possibly exposed to asbestos was significantly higher than in the unexposed control group

	Odds ratio	95% confidence interval	*P*-value
No lesion group/control	6.15	2.56–18.3	<0.001

significant differences in the detection rate of serum CCL3 between any of these groups. In contrast, the detection rate of CCL3 in the serum of the 10 patients constituting the mesothelioma group (9/10) was significantly higher than in the other groups (Table 5).

Immunohistochemical localization of CCL3 in malignant mesotheliomas. All biopsy specimens (4) and the autopsy specimen (1) showed clear expression of CCL3 in the tumor cells. In Figure 3, panel A is a malignant mesothelioma with glandular formation, and panel B is a malignant mesothelioma with solid proliferation. The tumor cells co-express CCL3 and mesothelin with CCL3 localizing primarily to the cytoplasm and mesothelin localizing more to the plasma membrane. These specimens were negative for the macrophage marker CD68 (data not shown). The levels of serum CCL3 of these two cases were 40.2 (panel A) and 2012.4 (panel B) pg/mL.

Discussion

Asbestos has a long history of use worldwide, and annual global production of asbestos remains at over 2 million tons.[3] The extensive use of asbestos has resulted in widespread risk of developing asbestos-associated diseases due to deposition of asbestos in the lung and pleural tissue, which can persist for the remainder of the exposed person's lifetime, causing foreign body inflammation in the lung and pleura. The ability to iden-

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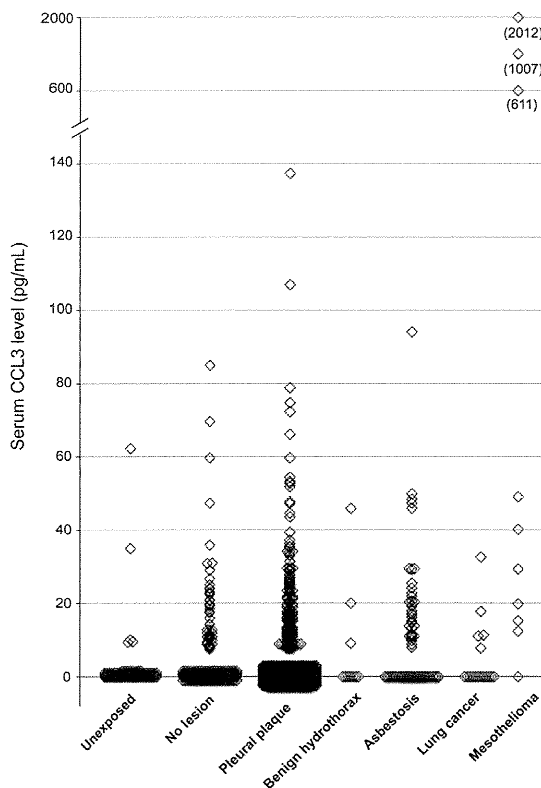

Fig. 2. Serum chemokine (C-C motif) ligand 3 (CCL3) levels in the secondary study group. Levels of CCL3 in study participants possibly exposed to asbestos and diagnosed with pleural plaque, benign hydrothorax, asbestosis, lung cancer, and mesothelioma are shown. For ease of comparison, the primary study group is shown alongside the secondary study group. The upper region of the graph is a log plot and is not continuous with the lower region of the graph. The serum CCL3 levels of the three patients plotted in the upper region are shown. Excluding the subjects without detectable CCL3 and the three subjects with exceptionally high levels of CCL3, there are no differences in CCL3 levels between any of the groups.

Table 5. Detection of serum chemokine (C-C motif) ligand 3 in the secondary study group, composed of subjects possibly exposed to asbestos and diagnosed with lung disease

Lesion category	Total number of subjects	Number of positive subjects	Detection rate, %	95% confidence interval
Exposed to asbestos				
Pleural plaque†	412	143	34.7	30.3–39.4
Asbestosis	86	34	39.5	29.9–50.1
Benign hydrothorax	10	3	30.0	10.8–60.3
Lung cancer	17	5	29.4	13.3–53.1
Malignant mesothelioma	10	9	90.0	59.6–98.2

†Includes 12 cases of pleural plaque with pneumoconiosis (mainly silicosis).

tify asymptomatic persons exposed to asbestos is obviously advantageous.

The purpose of the present study was to determine whether the levels or detection rate of CCL3 in the serum were elevated in asbestos-exposed individuals. In study subjects with detectable levels of serum CCL3, the levels were not elevated in asbestos-exposed subjects compared to unexposed subjects. In contrast to serum levels, the detection rate of CCL3 in the serum of healthy, asymptomatic subjects possibly exposed to asbestos was significantly higher than in unexposed healthy subjects: the detection rate in healthy, asymptomatic subjects possibly exposed to asbestos was 30.2% and the detection rate in healthy unexposed subjects was 6.6% (see Table 3). Serum CCL3 detection rate was independent of age, gender, smoking habit (never, previous, or current smoker), cigarette consumption (scored by the Brinkman index), the length of exposure time to asbestos, and the lapse of time after the last exposure to asbestos.

Although the detection rate of CCL3 in the serum of healthy, asymptomatic subjects possibly exposed to asbestos was significantly higher than in the serum of unexposed control subjects, a detection rate of 30% suggests that use of serum CCL3 as a biomarker for asbestos exposure is limited. Importantly, however, verification of the presence of asbestos fibers in the lung of a healthy asymptomatic person requires microscopic examination of biopsy or autopsy specimens; therefore, confirmation of the presence of asbestos fibers in the lung or pleural tissues of a healthy, asymptomatic person possibly exposed to asbestos is not carried out. Accordingly, healthy, asymptomatic study participants with certificates of asbestos exposure must be assumed to be possibly exposed to asbestos, resulting in the asbestos-exposed no lesion study group being composed of an above average number of individuals exposed to asbestos rather than being composed entirely of asbestos-exposed individuals, and it is very unlikely that all, or even most, of the subjects in the asbestos-exposed no lesion group actually have asbestos fibers in their lungs or pleural tissues. Consequently, the true percentage of these study participants with internalized asbestos fibers having detectable levels of serum CCL3 is unknown, but it is likely to be higher, probably much higher, than 30%. Therefore, detection of CCL3 in the serum could be a valuable adjunct marker for diagnosis of asbestos exposure.

The CCL3 chemokine is produced by macrophages and other immune cells in response to infection and various other insults; therefore, obtaining multiple blood samples from a patient is needed for proper evaluation of serum CCL3 levels. It is likely that if multiple samples are obtained, the detection of serum CCL3 in healthy unexposed subjects would decrease. As detection of serum CCL3 cannot distinguish between asbestos exposure and other disease states that result in increased levels of serum CCL3, use of CCL3 as an adjunct marker of asbestos exposure is appropriate only for asymptomatic individuals.

Frustrated phagocytosis of asbestos fibers is likely to play a role in asbestos-associated diseases.[51] In addition, our previous study in rats showed that intrapulmonary dosed nanoscale titanium dioxide particles were phagocytosed by alveolar macrophages and these macrophages secreted CCL3, resulting in elevated levels of CCL3 in the serum of rats treated with titanium dioxide.[49] These data suggest that the elevation of serum CCL3 in asbestos-exposed subjects is probably due to a persistent inflammatory reaction of macrophages interacting

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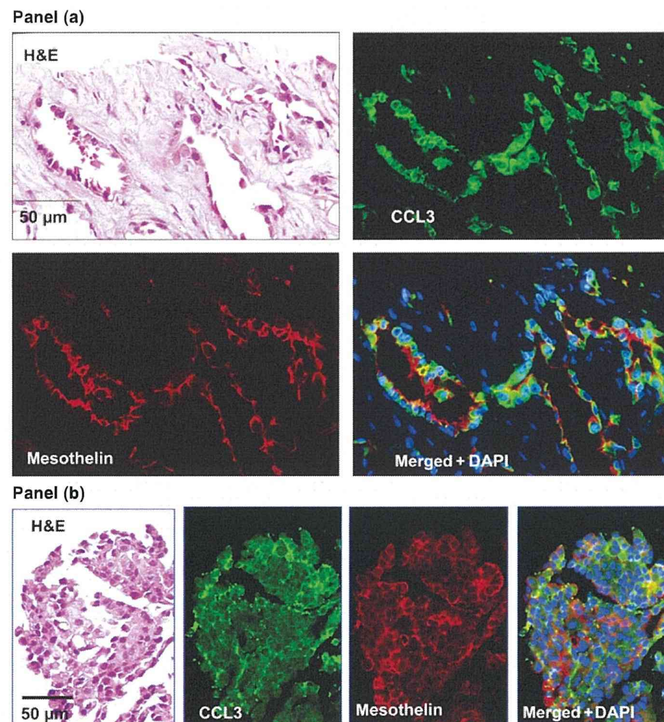

Fig. 3. Chemokine (C-C motif) ligand 3 (CCL3) and mesothelin in two biopsy specimens from patients with malignant mesothelioma. (a) Immunofluorescent staining of CCL3 and mesothelin in an enrolled biopsy case of malignant mesothelioma with glandular formation (serum CCL3, 40.2 pg/mL). Although CCL3 and mesothelin are coexpressed in the majority of tumor cells, the intracellular localization of CCL3 and mesothelin are distinct: mesothelin is localized primarily in the periphery of the tumor cells, whereas CCL3 is primarily cytoplasmic. (b) Immunofluorescent staining of CCL3 and mesothelin in an enrolled biopsy case of malignant mesothelioma with solid proliferation (serum CCL3, 2012.4 pg/mL). Similar to glandular-type malignant mesothelioma (a), most of the tumor cells expressed both CCL3 and mesothelin. Again, mesothelin is localized primarily at the cell periphery and CCL3 is more cytoplasmic.

with asbestos fibers even after the cessation of exposure to airborne asbestos.

Notably, the detection rate of CCL3 in the serum of the asbestos-exposed pleural plaque and asbestosis groups was not significantly different from the asbestos-exposed no lesion group. As the percentage of subjects diagnosed with pleural plaque and asbestosis that have asbestos fibers in their lung or pleural tissues is likely to be much higher than for the healthy, asymptomatic subjects possibly exposed to asbestos that constitute the asbestos-exposed no lesion group, this suggests that the detection rate of CCL3 in the serum of asbestos-exposed persons with certain asbestos-associated diseases is lower than for asymptomatic asbestos-exposed persons. One possible explanation for this seeming anomaly is that development of fibrotic tissue associated with inhaled asbestos fibers can prevent interaction of macrophages with the fibers.

Our study included 10 patients diagnosed with malignant mesothelioma. While this small number of malignant mesothelioma patients precludes assessment of CCL3 as a marker of malignant mesothelioma, two important observations were made. First, biopsy and autopsy specimens of malignant mesothelioma showed CCL3 expression by the tumor cells (see Fig. 3). Second, 9 of the 10 patients with malignant mesothelioma had detectable serum CCL3, and serum CCL3 levels in three of the malignant mesothelioma patients were dramatically higher than in the other study subjects. These observations suggest that expression of CCL3 by malignant mesothelioma tumor cells may be a source of the CCL3 in the serum of these patients and may result in extremely high levels of CCL3 in the serum of some malignant mesothelioma patients. We are currently accessing more malignant mesothelioma cases and appropriate controls to investigate the possibility that CCL3 may be a biomarker for malignant mesothelioma. The use of CCL3 in the pleural fluid and biopsy specimens for diagnosis of mesothelioma is also being examined.

In conclusion, inhalation of asbestos elicits a high risk of developing lung and mesothelial diseases, including fatal malignant mesothelioma. Careful follow-up of patients exposed to asbestos is a key issue in controlling the development of asbestos-associated diseases. Accordingly, identification of healthy, asymptomatic persons exposed to asbestos is an important goal. The CCL3 chemokine is detectable in the serum of a significant percentage of asymptomatic persons exposed to asbestos. Persistent detection over time of CCL3 in the serum of a healthy individual possibly exposed to asbestos

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can be used as an adjunct marker of asbestos exposure, and consequently as a risk marker for developing asbestos-associated diseases, including malignant mesothelioma. Including CCL3 in the standard blood tests for people at risk of asbestos exposure is likely to be of immediate and significant benefit.

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Disclosure Statement

Hiroyuki Tsuda has submitted a patent application for CCL3: #PCT /JP2012/056321. None of the other authors has a conflict of interest.

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Supporting Information

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

Table S1. Study participant data.

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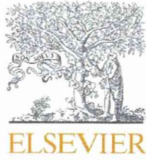

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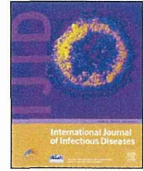

Dynamics of immune parameters during the treatment of active tuberculosis showing negative interferon gamma response at the time of diagnosis

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SUMMARY

Objectives: In the performance of interferon gamma release assays (IGRA) for the diagnosis of tuberculosis (TB) infection, false-negative results are a major obstacle. In active TB patients, treatment-dependent changes of the negative test results remain unknown.

Methods: The treatment course of 19 smear-positive/culture-confirmed TB patients who had IGRA-negative results by QuantiFERON-TB in-tube (QFT-IT) method at the time of diagnosis (month 0) in a previous study, were monitored in the present study. Blood was further collected at months 2 and 7, and the concentrations of 27 immune molecules were measured in the plasma supernatants remaining after performing the IGRA, using a suspension array system.

Results: After initiating treatment, eight of the 19 QFT-IT-negative patients showed positive conversion, whereas the remaining 11 (58%) did not; the interferon gamma (IFN-γ) response was restored to levels higher than 1 IU/ml in only three of the eight patients with positive conversion. Plasma concentrations of interleukin 1 receptor antagonist, interleukin 2, and interferon gamma-induced protein 10 remained low after *Mycobacterium tuberculosis*-specific antigen stimulation at months 2 and 7 in the continuously QFT-IT-negative group, whereas the parameters were elevated only in the transiently QFT-IT-negative group.

Conclusions: It was demonstrated that a majority of active TB patients showing negative IGRA results did not regain sufficient levels of immune responsiveness despite successful treatment.

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1. Introduction

The interferon gamma release assay (IGRA) is currently used as one of the representative tests to diagnose tuberculosis (TB) infection.[1] In this test, the cellular response to *Mycobacterium tuberculosis* is assessed by measuring the interferon gamma (IFN-γ) released from peripheral blood lymphocytes after stimulation with *M. tuberculosis*-specific antigens.[1]

The QuantiFERON-TB Gold In-Tube test (QFT-IT) is a commercially available IGRA based on the ELISA method; it has a sensitivity of 78–83% and specificity of 98–100%.[1] This imperfect sensitivity causes difficulties in ruling out TB infection, particularly when the

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prevalence of TB infection is high, and the low negative predictive value of the test may reduce the chance of a possible therapeutic intervention.

To assess the sensitivity of the QFT-IT, patients with bacteriologically proven active TB disease have often been recruited as surrogates for individuals with latent TB infection. The IGRA results also provide a clue to suspect active TB disease clinically.

Although weakened immunity in severe TB may affect the test results,[2] treatment-dependent changes of the negative IGRA results with a very low IFN-γ response have not been investigated fully.

This study group has recently reported the results of a cross-sectional study on the sensitivity of the QFT-IT method in Hanoi, Vietnam, and demonstrated that aging, emaciation, HIV co-infection, and a particular HLA genotype, DRB1*07:01, lowered the sensitivity of the test in active pulmonary TB patients.[3] In the present study, 19 of the 24 patients who showed false-negative results at the time of diagnosis were monitored. Further analysis of the treatment response and dynamics of immune parameters was performed, with the measurement of the concentrations of various cytokines and chemokines in the plasma supernatants remaining after use in the IGRA assay.

2. Methods

2.1. Study subjects and IGRA

From July 2007 to March 2009, whole blood was collected from 504 adult patients in Hanoi, Vietnam, who had smear-positive/culture-confirmed pulmonary TB and a history negative for TB treatment. The blood was collected in heparinized tubes before anti-TB treatment was initiated (month 0).[3] The patients were tested with a commercially available ELISA-based IGRA (QFT-IT; Cellestis, Victoria, Australia), as reported previously.[3] Plasma supernatants were separated at 4000 rpm for 15 min (Model 2010; Kubota Co., Tokyo, Japan) and stored at −80 °C until measurement. The cut-off value to interpret the QFT-IT results was set at 0.35 IU/ml, as per the manufacturer's instructions.

In the present study, further blood samples for QFT-IT and other tests were collected and served for analysis at two more time points: after the initial phase of treatment (month 2) and close to the end of treatment (month 7). Positive conversion of the IGRA was defined by a negative result at month 0 and positive result(s) at month 2, month 7, or both time points.

2.2. Clinical data collection

The extents of cavitary lesions and infiltrates were also semi-quantitated by the grading method.[4] *M. tuberculosis* isolates were analyzed by single nucleotide polymorphism (SNP) and spoligotyping methods.[5]

2.3. Treatment course

Following the national standard regimen at that time, all patients received an 8-month course of the anti-TB treatment regimen 2S(E)HRZ/6HE, which was commonly administered during the study period in Vietnam.[5]

2.4. Immune analyte profiling by Bio-Plex assay and adiponectin ELISA assay

Immune molecules released into the plasma after TB antigen stimulation were estimated from their concentrations after a 16- to 24-h incubation with TB-specific antigens (TBAg) minus those with no antigens (Nil) obtained from the QFT-IT method. Their concentrations were determined using a human 27-plex assay (14 cytokines: interleukin (IL)-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, tumor necrosis factor alpha (TNF-α); seven chemokines: eotaxin, IL-8, IFN-γ-inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory proteins MIP-1α and MIP-1β, RANTES; and six growth factors: IL-7, fibroblast growth factor (FGF) basic, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF)-BB, vascular endothelial growth factor VEGF) (Bio-Plex Suspension Array System; Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. All samples, standards, and controls were run in duplicate and manipulated in accordance with the manufacturer's protocol. Samples were diluted in a 1:4 volume ratio with the sample diluent and incubated for 30 min at room temperature; they were then agitated at 300 rpm to be captured with antibody-coupled magnetic beads. Following three washes in a Bio-Plex Pro Wash Station, the samples were incubated with biotinylated detection antibodies and agitated at 300 rpm in the dark for 30 min at room temperature. Each captured analyte was detected by the addition of streptavidin–phycoerythrin and quantified using a Bio-Plex array reader. The fluorescence intensities in the samples and known standards were acquired and converted to the plasma concentrations of each analyte using the Bio-Plex 200 System software (version 6.0; Bio-Rad Laboratories). When the induction of immune molecules after TB antigen stimulation was below their detection limits, these molecules were excluded from subsequent analysis.

Total human adiponectin (low, middle, and high molecular weight) levels in plasma were also measured using the Quantikine Human Total Adiponectin/Acrp30 Immunoassay Kit (R&D Systems, Inc., Minneapolis, MN, USA). The mean minimum detectable dose was 0.246 ng/ml.

2.5. Statistical analysis

Values including cytokine concentrations among groups were analyzed by Kruskal–Wallis tests with multiple comparisons for all pairs by Steel–Dwass method. The inequality of proportions among the groups was analyzed by Fisher's exact test. The statistical analysis was performed using Stata version 12 (Stata Corp, College Station, TX, USA) and JMP 9 (SAS Institute Inc., Cary, NC, USA). A p-value of < 0.05 was considered to be statistically significant. The Bonferroni correction was also used for multiple comparisons, when appropriate.

3. Results

3.1. Characteristics of the patients who completed the three-time blood collection stratified by IGRA-negative or positive result at month 0

After the cross-sectional study reported previously,[3] 19 of the 24 IGRA-negative patients with culture-confirmed active pulmonary TB at the time of diagnosis completed the three-time blood collection at months 0, 2, and 7; these patients were thus analyzed in the present study. The 351 patients who initially showed QFT-IT-positive results and completed the three-time blood collection were set as a reference. Samples showing indeterminate results before treatment were omitted from this analysis.

As expected from the results of the previous report,[3] increasing age, low body mass index (BMI) at the time of diagnosis, and the HLA-DRB1*07:01 allele were observed more frequently in the 19 QFT-IT-negative patients than in the 351 QFT-IT-positive

patients before treatment (**Supplementary Material**, Online Resource 1). HIV co-infection was nearly significantly associated with QFT-IT-negative results ($p = 0.0541$). In addition, it was possible to combine QFT-IT results with *M. tuberculosis* strain genotypes in 90% of these patients. Approximately 60% of the *M. tuberculosis* strains were of the Beijing genotype in this population, according to the previous results of SNP analysis and spoligotyping.[5] Non-Beijing strains, particularly EAI strains, were predominant in the QFT-IT-negative group as compared to the QFT-IT-positive group (52.9% vs. 18.3%; $p = 0.0004$; **Supplementary Material**, Online Resource 1).

3.2. Treatment course of the patients stratified by IGRA-negative or positive result at month 0

During treatment, QFT-IT IFN-γ values (TBAg minus Nil) of the 19 patients who showed QFT-IT-negative results at month 0 remained relatively low (Table 1).

The number of patients with a positive sputum smear was relatively small at month 7, indicating that a majority of the patients in both groups had been treated successfully; only one of 19 (5.3%) and five of 351 (1.4%) patients were regarded as treatment failure in the QFT-IT-negative and positive groups, respectively, at month 0, and this difference was not significant (data not shown). The extent of cavitary lesions and infiltrates on chest X-ray at month 0 and month 7 were also not different between the QFT-IT-negative and positive groups (Table 1).

3.3. Characteristics of the IGRA-negative patients at month 0 stratified by the absence or presence of positive conversion during treatment

Eight of the 19 QFT-IT-negative patients showed positive conversion at month 2 or month 7, whereas the remaining 11 (58%) did not. Background information, such as age and low BMI, was further compared between these two subgroups, but no particular characteristics were statistically significant (Table 2). Only three of the eight patients with positive conversion showed IFN-γ values higher than 1 IU/ml at month 2 (1.0, 2.2, and 3.2 IU/ml) and month 7 (1.8, 5.2, and 10.0 IU/ml). These patients did not carry any of the DRB1*07:01 alleles (data not shown), whereas all of the patients carrying the HLA-DRB1*07:01 allele in the QFT-IT-negative group showed IFN-γ values lower than 1.0 IU/ml throughout the treatment period (data not shown).

During treatment, sputum smear and chest X-ray findings were not significantly different between the subgroups with and without positive conversion. A trace of cavitary lesions was not observed in any of the five patients without positive conversion, but was observed in three of the six patients with positive conversion (**Supplementary Material**, Online Resource 2), although this difference was not statistically significant ($p = 0.18$).

3.4. Treatment-dependent changes in immunological parameters in the blood from patients with IGRA-negative results before treatment

The concentrations of immune molecules induced by *M. tuberculosis* antigen-specific antigens were analyzed in patients

Table 1
Treatment response of the patients stratified by QFT-IT-negative or positive result at month 0

		Negative result before treatment ($n = 19$)		Positive result before treatment ($n = 351$)		p-Value
QFT-IT-positive result	Month 0	0	(0.0%)	351	(100.0%)	<0.0001
	Month 2	5	(26.3%)	312	(88.9%)	<0.0001
	Month 7	6	(31.6%)	300	(85.5%)	<0.0001
QFT-IT value (IU/ml)	Month 0	0.21	(0.05–0.25)	8.61	(3.46–14.9)	<0.0001
	Month 2	0.11	(0.03–0.52)	3.80	(1.15–11.64)	<0.0001
	Month 7	0.19	(0.02–0.79)	2.85	(0.88–8.82)	<0.0001
Positive sputum smear	Month 0	19	(100.0%)	351	(100.0%)	1.0000
	Month 2	4	(21.1%)	40	(11.4%)	0.2623
	Month 5	1	(5.3%)	6	(1.7%)	0.3107
	Month 7	1	(5.3%)	5	(1.4%)	0.3107
Chest X-ray						
Extent of cavity (number of affected zones of the lung field)[a]	Month 0					0.6753
	0	5	(27.8%)	105	(31.2%)	
	1	9	(50.0%)	174	(51.6%)	
	2	3	(16.7%)	46	(13.7%)	
	3	1	(5.6%)	8	(2.4%)	
	>4	0	(0.0%)	4	(1.2%)	
	Month 7					0.1304
	0	8	(72.7%)	234	(91.8%)	
	1	3	(27.3%)	19	(7.5%)	
	2	0	(0.0%)	1	(0.4%)	
	3	0	(0.0%)	1	(0.4%)	
	>4	0	(0.0%)	0	(0.0%)	
Extent of infiltration (number of affected zones of the lung field)[b]	Month 0					0.1723
	0	2	(11.1%)	16	(4.7%)	
	1	3	(16.7%)	81	(23.9%)	
	2	4	(22.2%)	117	(34.5%)	
	3	3	(16.7%)	73	(21.5%)	
	>4	6	(33.3%)	52	(15.3%)	
	Month 7					0.3157
	0	3	(27.3%)	106	(41.7%)	
	1	4	(36.4%)	99	(39.0%)	
	2	4	(36.4%)	33	(13.0%)	
	3	0	(0.0%)	11	(4.3%)	
	>4	0	(0.0%)	5	(2.0%)	

QFT-IT, QuantiFERON-TB in-tube.
[a] $n = 18$ and $n = 337$ at month 0; $n = 11$ and $n = 255$ at month 7.
[b] $n = 18$ and $n = 339$ at month 0; $n = 11$ and $n = 254$ at month 7.

Table 2
Characteristics of QFT-IT-negative patients at month 0 stratified by the absence or presence of positive conversion

		With no positive conversion during treatment (n = 11)		With positive conversion during treatment (n = 8)		p-Value
Sex	Male	11	(100.0%)	7	(87.5%)	0.4211
	Female	0	(0.0%)	1	(12.5%)	
Age at diagnosis, years		55.4	(37.4–67.0)	43.3	(30.8–49.0)	0.1167
Body mass index, kg/m^2		16.7	(15.8–19.1)	15.9	(13.1–17.8)	0.3637
Smoking habit	Smoker/ex-smoker	8	(72.7%)	6	(75.0%)	1.0000
	Non-smoker	3	(27.3%)	2	(25.0%)	
BCG vaccination[a]	No	1	(9.1%)	1	(12.5%)	1.0000
	Yes	1	(9.1%)	0	(0.0%)	
	Unknown	9	(81.8%)	7	(87.5%)	
Sputum smear	Scanty	2	(18.2%)	0	(0.0%)	0.7611
	+	4	(36.3%)	4	(50.0%)	
	++	3	(27.3%)	3	(37.5%)	
	+++	2	(18.2%)	1	(12.5%)	
HIV status	Negative	9	(81.8%)	7	(87.5%)	1.0000
	Positive	2	(18.2%)	1	(12.5%)	
White blood cell count $\times 10^9$/l		8.6	(5.3–12.0)	9.9	(9.5–18.8)	0.2151
Lymphocyte count $\times 10^9$/l		1.4	(0.8–2.0)	2.1	(1.7–2.5)	0.1345
Drug resistance	Isoniazid	1	(9.1%)	1	(12.5%)	1.0000
	Rifampicin	1	(9.1%)	0	(0.0%)	1.0000
	Streptomycin	2	(18.2%)	1	(12.5%)	1.0000
	Ethambutol	1	(9.1%)	0	(0.0%)	1.0000
HLA-DRB1*07:01 allele number	0	6	(54.5%)	6	(75.0%)	0.1852
	1	4	(36.4%)	0	(0.0%)	
	2	1	(9.1%)	2	(25.0%)	
MTB strains[b]	Beijing	1	(11.7%)	2	(25.0%)	0.5335
	EAI	6	(66.7%)	3	(37.5%)	
	Other	2	(22.2%)	3	(37.5%)	

QFT-IT, QuantiFERON-TB in-tube; BCG, bacille Calmette–Guérin; MTB, *Mycobacterium tuberculosis*.
[a] Self-declaration.
[b] n = 9 and n = 8.

showing negative results at month 0 with positive conversion at months 2 or 7, in patients showing negative results at month 0 without positive conversion, and in 21 randomly selected patients who showed a QFT-IT-positive result at month 0, and then completed the treatment course.

Of the 27 immune analytes in the plasma supernatants, IL-1RA, IL-2, IFN-γ, and IP-10 showed differences in concentrations among the groups, even after Bonferroni correction; these immunological parameters were predominantly induced in the QFT-IT-positive reference group, whereas their concentrations in QFT-IT-negative subgroups were low (Figure 1). Although the induction levels of these immune parameters gradually decreased during treatment in the QFT-IT-positive group, significant differences remained between the QFT-IT-positive group and the continuously negative group without positive conversion. In the transiently negative group with positive conversion, levels of these immune parameters were restored to some extent during the course of treatment; concentrations of IL-1RA and IL-2 in the transiently negative group were significantly higher than those in the continuously negative group at month 7 (Figure 1). Concentrations of IL-10, known as a regulatory cytokine, were not significantly different between these two subgroups; adiponectin concentrations did not show a significant difference either (data not shown).

4. Discussion

The QFT-IT test results of Vietnamese patients with culture-confirmed active pulmonary TB enrolled in a cross-sectional study were recently reported,[3] and factors that possibly lower the sensitivity of this ELISA-based IGRA were identified: aging, emaciation, HIV co-infection, and a particular HLA genotype, DRB1*07:01.[3] In the present study, the IGRA-negative TB patients from the previous study underwent continued monitoring during treatment, and treatment-dependent changes in immune status were characterized by measuring the concentrations of cytokines and chemokines in the plasma supernatants.

IL-1RA, IL-2, and IP-10, as well as IFN-γ, were more or less inducible after TB antigen-specific stimulation (TBAg minus Nil) in active pulmonary TB. The induction levels were significantly lower in the continuously IGRA-negative group than in the IGRA-positive group, even after initiating treatment. This is possibly due to immune regulatory mechanism(s), including impaired antigen presentation through a particular HLA molecule or long-lasting T-cell anergy,[6,7] although immunoregulatory IL-10 levels relevant to the regulatory T-cells were not different between these groups.

The difference in cytokine/chemokine induction was gradually lost between the IGRA-positive group and the transiently IGRA-negative group. The immune dynamics in the patients in whom the immune response was restored may be consistent with those of an early report, which showed the suppression of IFN-γ production from CD4+ T-cells in response to TB-specific peptides in patients with severe pulmonary TB before treatment.[2] However, the IFN-γ response was fully restored (>1 IU/ml) during the treatment period in only three of the 19 patients (16%).

It has thus been demonstrated that IGRA-negative patients tend to have continuously low immune responsiveness despite successful treatment, measuring not only IFN-γ but also other immune molecules. These findings indicate that factors unaffected by treatment are important when negative IGRA results are interpreted in active TB.

IL-1RA is a natural antagonist of IL-1 produced by activated monocyte/macrophages, and blocks the binding of IL-1α and IL-1β to the type I IL-1 receptor, without exerting agonist activity. IL-1RA is elevated in the blood of patients with inflammation, and IL-1RA has been suggested as a marker for TB disease activity and response to treatment.[8] Increased levels of IL-1RA were found both in the lung epithelial lining fluid and in the blood from patients with active pulmonary TB.[9] IL-1 is not easily detected in the circulation during human disease, and IL-1RA may be a better maker than IL-1 as an indicator of ongoing inflammation.[10]

The restoration of IL-2 production in the IGRA-negative group with positive conversion is consistent with previous reports:[11,12]

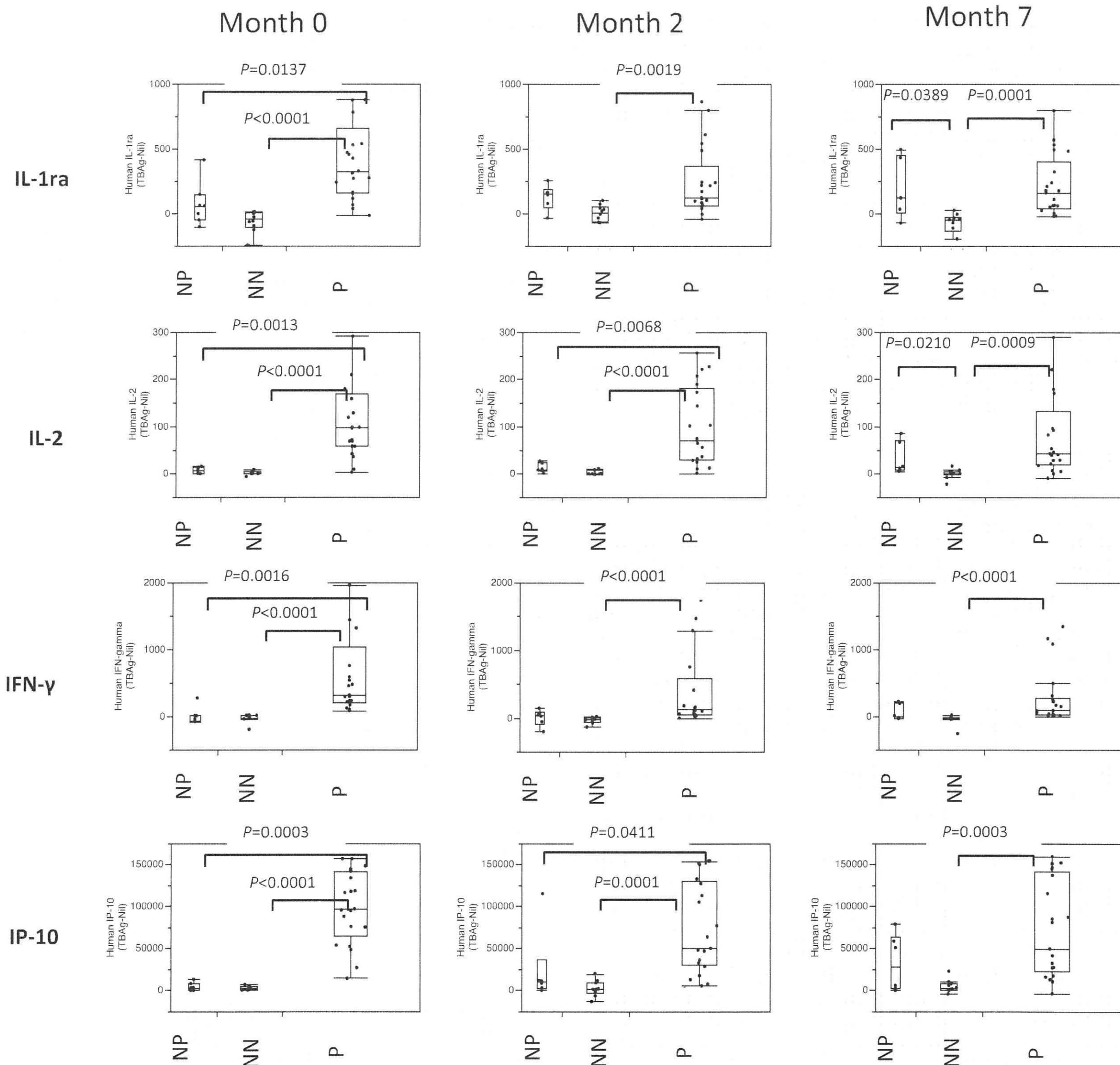

Figure 1. Immune analyte concentrations after TB antigen-specific stimulation at months 0, 2, and 7. Immune analyte concentrations (ng/ml) after TB antigen-specific stimulation (TBAg minus Nil) were compared among IGRA-negative groups at month 0 with positive conversion at months 2 or 7 (NP) and without positive conversion (NN), and the IGRA-positive reference group at month 0 (P). A significant difference in IL-1RA, IL-2, IFN-γ, and IL-2 concentrations was observed even after Bonferroni correction (uncorrected $p < 0.0005$ by Kruskal–Wallis test). All pairs were further compared by Steel–Dwass method, and their significant p-values are shown.

in patients with normal TB immunity, it is known that antigen-specific IFN-γ-only-secreting effector T-cells are predominant before treatment and dual IFN-γ/IL-2-secreting or polyfunctional T-cells with memory cell characteristics become predominant after starting treatment.[11,12] In the continuously IGRA-negative group, effector memory T-cells might have failed to expand for unknown reasons. Indeed, when the suspension array data were analyzed, low IL-2 induction in QFT-IT was observed together with the low IFN-γ response in the patients without positive conversion.

IP-10 is a chemokine expressed by antigen-presenting cells in response to IFN-γ, and IP-10-based tests are comparable with the IGRA response[13] before and during treatment.[14] This study group has previously reported that circulating adiponectin may be a marker for the severity of the disease,[15] but this parameter was not clearly associated with IGRA results in the present study.

Changes in clinical phenotypes relevant to treatment failure, such as chest radiographic lesions and smear-negative conversion, were not associated with the IGRA results at month 0. In the present study, the transiently IGRA-negative group with positive conversion did not show any clinical backgrounds distinct from those of the continuously IGRA-negative group without positive conversion, presumably because disease severity is further affected by the combination of other factors including patient age, patient BMI, and the bacterial burden.

Furthermore, *M. tuberculosis* strains of non-Beijing EAI genotype were frequently observed in the IGRA-negative group in this study. This may be partly confounded by the fact that non-Beijing strains are often observed in elderly people in Hanoi.[16] However, confirmation based on multivariable analysis was not performed because of the small sample size.

The HLA-DRB1*07:01 allele, an endogenous genetic factor, has previously been reported to be associated with QFT-IT false-negative results or a low IFN-γ response.[3] This tendency was shown throughout the treatment course in the present study, although it did not reach statistical significance, presumably due to the relatively small sample size.

These findings will provide insights into factors affecting QFT-IT false-negative results over a long term, although they may not be extrapolated to those of another IGRA, the T-SPOT.TB. Nevertheless, a variety of factors that potentially suppress the IGRA response should be investigated further and the test performance of the IGRA should be improved in future studies.

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Ethics statement: The study was approved by the ethics committees of the Ministry of Health, Vietnam, National Center for Global Health and Medicine, and The Research Institute of Tuberculosis, JATA Japan. All participants were enrolled upon provision of written informed consent.

Conflict of interest: The authors have declared that no competing interests exist.

Author contributions: IM carried out the immunoassays, drafting the paper. NTLH participated in supervising the on-site implementation of the study. LTH and DBT carried out the immunoassays. LTL and PHT participated in conception, design and supervision of the study. VCC participated in supervising the on-site implementation of the study. MH participated in analysis and interpretation of the data. NKo and SS participated in conception and design of the study. KH and NH were responsible for technical transfer and supervision. NK was responsible for the conception, design and overall supervision of the study, analysis and interpretation of data, drafting the paper or substantially revising it. All authors read and approved the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2015.09.021.

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