by diminishing the recruitment of inflammatory TH17 cells via interferon-γ secreted by the $CD56^{bright}CD27^{+}$ NK cell subset. This NK cell–mediated regulatory response is lost in patients with recurrent spontaneous abortions, resulting in a prominent TH17 response and extensive local inflammation.

We previously reported (9) that elevated preconceptional peripheral blood NK (pNK) cell activity may be predictive of subsequent miscarriage in 68 patients with RPL. The 24 women with high pNK cell activity, defined as a value equal to or exceeding the mean pNK cell activity of the 47 controls + 1 SD, had a significantly higher miscarriage rate in the subsequent pregnancy than the 44 women with normal pNK cell activity (71% vs. 20%; relative risk 3.5; 95% confidence interval [CI] 1.8–6.5). However, some studies have shown no differences in pNK cell parameters between patients with RPL and controls (10–12). Whereas 90% of pNK cells are $CD56^{dim}$ and $CD16^{+}$, 80% of the uterine NK cells are $CD56^{bright}$ and $CD16^{-}$ (13, 14). Peripheral blood NK cells are phenotypically and functionally different from uterine NK cells. Decidual leukocytes have low cytotoxic activity as compared with peripheral lymphocytes (15).

The sample size of our previous study was too small. The prognostic value of measuring pNK cell parameters remains uncertain (16). Therefore, we conducted a cohort study of 552 patients with RPL to determine whether pNK might indeed be predictive of subsequent miscarriage.

MATERIALS AND METHODS

Patients

We studied 1,127 patients with a history of two or more (2 to 12) consecutive miscarriages, in whom the study examinations could be completed and subsequent pregnancies were established between January 1996 and May 2011 in Nagoya City University Hospital. Patients with identifiable causes and patients who received any kinds of treatment were excluded from the present cohort.

The conventional examinations were completed in all patients, including hysterosalpingography, transvaginal ultrasonography, chromosomal analysis of both partners, determination of the presence/absence of antiphospholipid antibodies, including lupus anticoagulant (LA), by diluted activated partial thromboplastin time (aPTT), diluted Russel viper venom time (RVVT), and β2 glycoprotein I–dependent anticardiolipin antibody methods (17), and blood tests for hypothyroidism and diabetes mellitus, before the subsequent pregnancy.

Antiphospholipid antibody syndrome (APS) was diagnosed according to the criteria of the International Congress on Antiphospholipid Antibodies (18). Patients with APS were treated with low-dose aspirin plus heparin (19). Diabetes mellitus, hyper- or hypothyroidism, and hyperprolactinemia in the patients were controlled with medication before conception. Patients with three or more unexplained miscarriages received paternal mononuclear cell immunization from 1996 to 1999, a biologic response modifier from 1996 to 2004 (20), and low-dose aspirin and/or heparin from 2000 to 2007.

Gestational age was calculated from basal body temperature charts. A total of 654 patients were admitted for rest, and ultrasonography was performed twice per week from 4 to 8 weeks of gestation before 2004. The pregnancy outcome of 473 patients was followed once per week by ultrasonography without admission after 2004.

The study was conducted with the approval of the Research Ethics Committee at Nagoya City University Medical School.

Measurement

Preconceptional pNK cell activity was examined in the midsecretory phase. Peripheral blood NK cell activity was measured by a chromium-51 release cytotoxicity assay, with K562 human myeloid leukemia cells as the targets. A total of 3.7×10^3 Bq ^{51}Cr-labeled target cells (1×10^4 per well) were seeded with 2×10^5 effector cells per well (fresh peripheral blood mononuclear cells) in triplicate, in U-bottomed 96-well plates. After 4-hour incubation at 37°C, the activity in the supernatant from each well was measured in an autogramma scintillation counter. The percentage cytotoxicity was calculated as follows: ([test cpm−spontaneous cpm]/[maximum cpm−spontaneous cpm]) × 100, where cpm = counts per minute.

Lupus anticoagulant was detected using fivefold diluted aPTT methods, as previously described, with brain cephalin (Automated aPTT; Organon Teknica) as the phospholipid reagent (17). The 1:1 mixing test was performed at the same time. The clotting time was measured using an Option 4 Biomerieux calculator. Lupus anticoagulant was considered positive when prolonged clotting times (>mean + 3 SD of 104 healthy nonpregnant control plasma, 7.37 seconds) failed to correct when mixed 1:1 with standard plasma. Diluted Russel viper venom time for LA was performed as previously described (Gradipore). To detect β2 glycoprotein I–dependent anticardiolipin antibody, we used a modified ELISA system (Yamasa).

Analysis

Patients with identifiable causes and patients who received any kinds of treatments were excluded from the present cohort. Biochemical pregnancy, ectopic pregnancy, and hydatidiform mole were excluded from the analysis of the subsequent pregnancy outcome.

Miscarriage rate was analyzed according to pNK cell activity, age, number of previous miscarriages, and presence/absence of previous live births and bed rest. Peripheral blood NK cells, age, and previous number of miscarriages were categorized into quartiles, because they showed normal distribution. The previous number of miscarriages was categorized into two, three, four, or five to six.

Crude logistic regression was performed to examine the predictive value of pNK cell activity for subsequent miscarriage. We also examined the influence of age, previous number of miscarriages, and presence/absence of previous live birth and bed rest on the likelihood of subsequent miscarriage.

Age is well known to influence the miscarriage rate. Age is also associated with number of previous miscarriages and number of previous live births. Thus, first, we chose

age-adjusted logistic regression. This analysis was applied to all the variables listed in the table except age.

Furthermore, multivariable logistic regression analysis was performed using pNK cell activity, age, number of previous miscarriages, and presence/absence of previous live births and bed rest as covariates. Linear multivariable logistic regression analysis was also performed using pNK cell activity, age, and number of previous miscarriages.

The analysis was carried out using SAS version 19.0 (SAS Institute), and $P<.05$ was considered to denote statistical significance.

RESULTS

In the subjected 1,127 patients, 4.4% (50) had an abnormal chromosome in either partner, 4.1% (46) of patients had a major uterine anomaly, 3.4% (38) had thyroid disease, 1.9% (21) had diabetes mellitus, and 2.9% (33) had APS (Fig. 1). In total, 180 patients were excluded from the cohort because several patients had two or three identifiable causes.

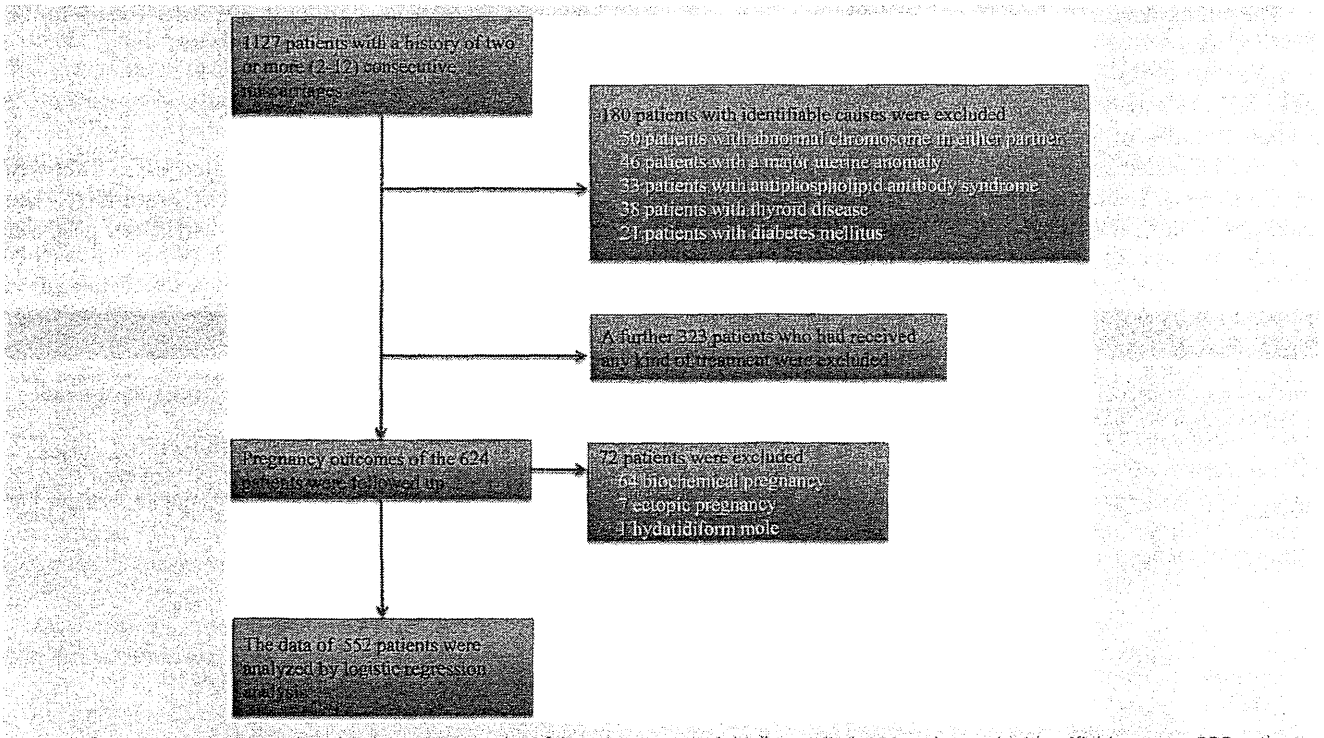
To eliminate the influence of the treatment, a further 323 patients who received any kind of treatment were excluded. A total of 72 patients—64 biochemical pregnancy, 7 ectopic pregnancy, and 1 hydatidiform mole—were excluded in the present study (Fig. 1).

The miscarriage rate of a total of 552 patients with unexplained RPL who received no medication is shown in Table 1. The subsequent miscarriage rate was 22.5% (124 of 552). The mean (SD) age and median (interquartile range) number of previous miscarriages were 31.9 (4.37) and 2 (2–3), respectively. The live birth rates of patients with previous two, three, four, five, and six miscarriages were 81.1% (309 of 381), 71.2% (99 of 139), 65.4% (17 of 26), 60.0% (3 of 5), and 0 (0 of 1), respectively.

Linear multivariable logistic regression showed that pNK was not an independent risk factor for subsequent miscarriage. However, in the crude analysis of the categorization of each variable, the miscarriage rate in the patients with 5%–24% pNK cell activity was significantly higher than that in the patients with 25%–34% pNK cell activity ($P=.046$). On the other hand, the miscarriage rate in the patients with 47%–78% pNK cell activity was similar to that in the patients with 25%–34% pNK cell activity. The plasma NK cell activity showed a weak inverse correlation with age in the 1,127 patients ($r = -0.068$).

Five variables, namely pNK cell activity, age, number of previous miscarriages, and absence of bed rest and previous live birth, were entered into the multiple logistic regression analysis for subsequent miscarriage detection in all 552 patients. The miscarriage rate in patients with 25%–34% pNK cell activity tended to be higher than that in patients with 5%–24% pNK cell activity (odds ratio [OR] 0.56, 95% confidence interval [CI] 0.31–1.00, $P=.051$). Crude, age-adjusted, and multivariable logistic regression analyses showed similar results in relation to pNK cell activity. Elevated pNK cell activity was confirmed to not be an independent risk factor for a subsequent miscarriage.

FIGURE 1



A total of 552 patients were analyzed in the present study. Of the 1,127 women initially enrolled, 180 patients with identifiable causes, 323 patients who received any kind of medication, and 72 patients whose pregnancy outcomes were biochemical or ectopic pregnancy were excluded.

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TABLE 1

Miscarriage rate according to pNK cell activity, age, and number of previous miscarriages, and age-adjusted and multivariable logistic regression analysis to identify the risk factors for subsequent miscarriage.

		Crude analysis logistic regression		Age-adjusted logistic regression[a]		Multivariable logistic regression[b]		
Parameter	Miscarriage rate, % (n)	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	Trend *P* value
Peripheral NK cell activity (%)								.365
5–24	28.1 (41/146)	Reference		Reference		Reference		
25–34	17.9 (24/134)	0.56 (0.32–1.00)	.046	0.55 (0.31–0.98)	.042	0.56 (0.31–1.00)	.051	
35–46	22.6 (31/137)	0.75 (0.44–1.28)	.293	0.73 (0.42–1.26)	.261	0.78 (0.45–1.36)	.385	
47–74	20.7 (28/135)	0.67 (0.39–1.16)	.154	0.69 (0.39–1.20)	.186	0.73 (0.41–1.30)	.282	
Age (y)								.0002
19–29	13.2 (22/167)	Reference				Reference		
30–31	28.7 (33/115)	2.65 (1.45–4.85)	.0015			2.49 (1.35–4.59)	.0036	
32–35	20.9 (33/158)	1.74 (0.96–3.14)	.0658			1.46 (0.79–2.71)	.226	
36–45	32.1 (36/112)	3.12 (1.72–5.68)	.0002			2.54 (1.35–4.76)	.0037	
No. of previous miscarriages								.0014
2	18.9 (72/381)	Reference		Reference		Reference		
3	28.8 (40/139)	1.73 (1.11–2.71)	.012	1.57 (0.99–2.48)	.055	1.38 (0.85–2.26)	.198	
4	34.6 (9/26)	2.27 (0.97–5.30)	.052	1.84 (0.77–4.37)	.168	1.65 (0.67–1.10)	.280	
5–6	50.0 (3/6)	4.29 (0.85–21.70)	.090	3.56 (0.68–18.55)	.132	3.73 (0.69–20.10)	.126	

[a] The only covariate used was age for the age-adjusted logistic regression analysis. This analysis was applied to all the variables listed in the table except age.
[b] The covariates used for the multivariable logistic regression analysis were pNK activity, age, number of previous miscarriages, presence/absence of previous live births, and presence/absence of bed rest.

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The miscarriage rate in the patients without bed rest was significantly higher than that in the patients who were admitted for rest ($P=.016$; Table 2). However, there were differences in the mean [SD] age (30.9 [4.0] vs. 32.8 [4.5] years; $P<.0001$) and the median number of previous miscarriages (three vs. two) between the patients with and without bed rest.

The miscarriage rate in patients without previous live births tended to be lower than that in the patients with previous live births ($P=.096$). There were differences in the mean age (33.9 [3.7] vs. 31.6 [4.4] years; $P<.0001$) and the median number of previous miscarriages (three vs. two) between the patients with and without previous live births.

No effect of bed rest and previous live birth on the likelihood of live birth was observed (OR 1.28, 95% CI 0.81–2.02 and OR 0.91, 95% CI 0.52–1.59, respectively).

Age and number of previous miscarriages were determined to be risk factors for subsequent miscarriage according to both crude and linear multivariable logistic regression.

Age and numbers of previous miscarriages were confirmed to be independent risk factors. However, number of previous miscarriage, but not age, was found to be influenced by the other factors in the present study.

DISCUSSION

The results of this study suggest that elevated pNK cell activity is not a reliable predictor of subsequent miscarriage. The miscarriage rate was higher in patients with lower pNK cell activity.

TABLE 2

Miscarriage rate according to the presence/absence of previous live births and bed rest, and age-adjusted and multivariable logistic regression analysis to identify the risk factors for subsequent miscarriage.

				Crude analysis logistic regression		Age-adjusted logistic regression[a]		Multivariable logistic regression[b]	
Parameter	Miscarriage rate, % (n)	Mean (SD) age (y)	Median no. of previous miscarriage	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Absence of previous live birth									
Presence	28.4 (25/88)*	33.9 (3.7)*	3*	Reference		Reference		Reference	
Absence	21.3 (99/464)*	31.6 (4.4)*	2*	0.68 (0.41–1.14)	.096	0.68 (0.41–1.14)	.146	0.91 (0.52–1.59)	.736
Absence of bed rest									
Presence	18.2 (47/258)*	30.9 (4.0)*	3*	Reference		Reference		Reference	
Absence	26.2 (77/294)*	32.8 (4.5)*	2*	1.59 (1.06–2.40)	.016	1.59 (1.06–2)	.026	1.28 (0.81–2.02)	.288

[a] The only covariate used was age for the age-adjusted logistic regression analysis. This analysis was applied to all the variables listed in the table except age.
[b] The covariates used for the multivariable logistic regression analysis were pNK activity, age, number of previous miscarriages, presence/absence of previous live births, and presence/absence of bed rest.
* $P<.05$ was considered to denote statistical significance.

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Uterine endometrial NK (uNK) cell activity is known to be strongly involved in the maintenance of normal pregnancy. Lachapelle et al. (21) proved that the proportion of uNK cells was identical in recurrent miscarriage (RM) patients and normal controls, but the $CD56^{bright}$ and $CD16^{-}$ NK cell subset, which is predominant in normal decidua and endometrium, was significantly decreased in favor of an important contingent of $CD56^{dim}$ and $CD16^{+}$ NK cells in all patients. Quenby et al. (22) demonstrated that prednisolone therapy during the first trimester of pregnancy reduced the risk of miscarriages and improved the live birth rate in patients with idiopathic RM and increased the numbers of uNK cells in the endometrium. Measurement of pNK cell activity has been performed to determine whether it might be predictive of a successful subsequent pregnancy (23, 24). We have reported for the first time that elevated pNK cell activity might be predictive of subsequent miscarriage in patients with RM (9). Some have affirmed, whereas others have denied, the predictive value of pNK for the subsequent pregnancy outcome. However, none of these reports were based on studies of large cohorts, and there is no clear evidence yet (16, 25).

Patients with unexplained RM have been treated empirically with expensive immunoglobulin, on the basis of the conjecture that the functions of uNK cells and pNK cells are similar and that, therefore, measurement of pNK cell activity would reflect uNK cell activity. Tang et al. (16) reported a systematic review and came to the conclusion that there is no association between the subsequent pregnancy outcome and either pNK or uNK cell activity in women with RM and infertility. In the present study the correlation between the subsequent pregnancy outcome and pNK cell activity was not linear. The miscarriage rate in patients with low pNK cell activity tended to be higher than that in patients with 25%–74% pNK cell activity. Age, number of previous miscarriages, bed rest, and number of previous live births were found to exert no significant influence on pNK cell activity.

It is well known that stress and exercise increase pNK cell activity; therefore, these factors should be borne in mind while drawing blood for testing. Abnormal data pertaining to the number or activity of pNK cells may reflect transient stress reactions in daily life. It is not clear whether uNK cells may have the same significance. Peripheral blood NK and uNK cells are different types of cells, and both the models and functions of these cells are entirely different. It has been reported that measurement of pNK cell activity does not provide any information on the condition of the endometrial membrane (7, 14, 25). There is also no evidence of treatment using the data on pNK cell activity. We do not recommend measurement of pNK cell activity as part of the systematic examination in patients with RPL.

Mentally depressed patients with RPL need tender loving care (26). However, there is no evidence that subsequent miscarriage can be prevented by hospitalization. Klebanoff et al. (27) concluded that there was no difference in the miscarriage rate between women who had a heavy workload and long working hours and wives of male residents who had many kinds of jobs. Duckitt et al. (28) found no direct evidence from randomized, controlled trials regarding the influence of bed rest in women with unexplained RM. In the present study the live birth rate in patients without bed rest was significantly lower than that in the patients who were admitted for rest. However, there were significant differences in the mean age and median number of previous miscarriages between the patients with and without bed rest, because the average age of women at pregnancy is increasing year by year in Japan. Neither age-adjusted nor multivariable logistic regression analysis showed any effect of bed rest on the live birth rate. We concluded that there is no necessity to advise preventive bed rest for pregnant women, in the absence of symptoms of threatened abortion.

This study revealed that previous live birth was not predictive of a subsequent live birth, although there have been a few reports suggesting a favorable influence of a previous live birth in secondary RM patients (29, 30). Nielsen (31) reported that secondary RM is more common after the birth of a boy and that the subsequent live birth rate is reduced in secondary RM patients with a firstborn boy, owing to the pathogenic role of the aberrant maternal H-Y immune response. Both our previous study and the crude analysis in the present study indicated that the live birth rate increased as the number of previous miscarriages increased (32). However, the significant difference disappeared after adjustment for age, because age also increased with increasing number of previous miscarriages.

In more than half of the cases, the cause of RPL remains unexplained despite conventional examinations (4, 5). Recently we found that an abnormal embryonic karyotype was the most frequent cause, accounting for as much as 41% of the cases, and the percentage of truly unexplained was limited to 24.5% (33). Associations have been reported between many kinds of polymorphisms, such as those of annexin A5 and NLRP7, and RPL (34, 35). The influence of one single-nucleotide polymorphism associated with RPL might be speculated to be very small, because the OR of each gene mutation is relatively small (34). Even though it would be highly desirable, it might be difficult to identify clinically useful predictors of the outcome of a subsequent pregnancy.

We previously reported that elevated pNK cell activity may be predictive of subsequent miscarriage in patients with RPL (9). However, we wish to correct our initial conclusion, because in this study, high pNK cell activity was confirmed to not be an independent risk factor for subsequent miscarriage. Clinicians should not measure pNK activity as a systematic RPL examination, because the clinical significance or treatment method is yet to be established. Patients need not give up working, because no effect of bed rest on the likelihood of live birth was observed.

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ORIGINAL ARTICLE

Elevation of KL-6 serum levels in clinical trials of tumor necrosis factor inhibitors in patients with rheumatoid arthritis: a report from the Japan College of Rheumatology Ad Hoc Committee for Safety of Biological DMARDs

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Abstract
Objective The associations between elevated levels of serum Krebs von den Lungen-6 (KL-6) and treatment of rheumatoid arthritis (RA) with tumor necrosis factor (TNF) inhibitors were investigated in five Japanese clinical trials.

Methods Percentages and incidence rates were calculated for elevated serum KL-6 levels. Adverse events associated with elevated levels of serum KL-6 were investigated.
Results In RISING, a clinical trial for infliximab, 15.6 % of the enrolled patients met criterion B (KL-6 ≥500 U/ml and >1.5-fold increase over the baseline value) by week 54. In HIKARI, 7.8 % of the certolizumab pegol (CZP) group and 0 % of the placebo group met criterion B during the double-blind (DB) period ($p = 0.003$). In J-RAPID, 8.4 % of the methotrexate (MTX) + CZP and

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3.9 % of the MTX + placebo groups met criterion B during the DB period. In GO-MONO, 1.8 % of the golimumab (GLM) and 1.3 % of the placebo groups met criterion B during the DB period. In GO-FORTH, 7.1 % of the MTX + GLM and 0 % of the MTX + placebo groups met criteron B during the DB period ($p = 0.017$). No adverse events accompanied the elevation of serum KL-6 levels in 95.7 % of these patients.

Conclusion Serum KL-6 levels may increase during anti-TNF therapy without significant clinical events. In these patients, continuing treatment with TNF inhibitors under careful observation is a reasonable option.

Keywords Biological disease modifying antirheumatic drug · KL-6 · Rheumatoid arthritis · Interstitial pneumonia · *Pneumocystis jirovecii* pneumonia

Introduction

During the last decade, the introduction of tumor necrosis factor (TNF) inhibitors for the treatment of rheumatoid arthritis (RA) has completely changed the treatment strategy and management of this intractable disease. In Japan, four TNF inhibitors have been approved for the treatment of RA and are widely used in clinical practice: infliximab (IFX) in 2003, etanercept (ETN) in 2005, adalimumab (ADA) in 2008, and golimumab (GLM) in 2011. Certolizumab pegol (CZP) is now under clinical development, and phase 3 and phase 2/3 trials have already been completed. For IFX, ETN, and ADA, post-marketing surveillance (PMS) programs have revealed short-term safety profiles of these biological disease-modifying antirheumatic drugs (DMARDs) in Japanese RA patients [1, 2]. Infection was the most frequently reported adverse drug reaction for IFX and ETN, and the second most for ADA. About half of these infectious events developed in the respiratory system. The results of the PMS and other clinical studies indicated that clinically important pulmonary infections in Japanese RA patients given TNF inhibitors encompassed bacterial pneumonia, tuberculosis, and *Pneumocystis jirovecii* pneumonia (PCP) [1–4].

The Krebs von den Lungen-6 (KL-6) antigen is a mucinous high-molecular-weight glycoprotein primarily derived from a lung adenocarcinoma cell line and classified as a cluster 9 mucin-1 of lung tumors and differentiation antigens [5]. KL-6 is produced by type II alveolar epithelial cells and is reported to be elevated in patients with idiopathic interstitial pneumonia (IIP), interstitial pneumonia (IP) associated with collagen diseases, other interstitial lung diseases, PCP, and malignancies [6–14]. Among the clinical trials for biological DMARDs, the "impact on Radiographic and clinical response of Infliximab therapy concomitant with methotrexate in patients with rheumatoid arthritis by the trough Serum level in the dose-escalatING study" (the RISING study) [15] systematically measured serum KL-6 levels for the first time. In a report to the Pharmaceuticals and Medical Devices Agency of Japan, the RISING study describes its findings of an abnormal elevation of this serum marker in RA patients receiving IFX without any development or exacerbation of pulmonary disease or malignancies. However, no peer review journal report of the details of the elevation of serum KL-6 has been published, and it has not been determined whether this adverse event is truly related to treatment with IFX, is common among treatment with TNF inhibitors or other biological DMARDs, or is related to treatment with MTX. A report of elevated serum KL-6 levels in three RA patients treated with ADA has been recently published [16].

In Japan, the measurement of serum KL-6 levels is an officially approved and widely used clinical laboratory test in the field of rheumatology. The Japan College of Rheumatology convened an ad hoc committee for the safety of biological DMARDs to investigate the abnormal elevation of serum KL-6 levels in RA patients given biological DMARDs. The committee implemented two studies to investigate this issue, one for clinical trial data and the other for clinical practice data. The results from the analyses of the clinical trial data are reported here; those from the study of clinical practice data will be reported separately.

Patients and methods

Clinical trials

Serum KL-6 levels were measured in clinical trials in Japan for three TNF inhibitors, IFX, CZP, and GLM. For our analyses, we utilized the RISING study for IFX [15], a pHase 3 study to assess the effIcacy, safety and phamacoKinetics of CDP870 (CZP) in rheumatoid ArthRItis patients (HIKARI; ClininalTrials.gov, NCT00791921) and the Japanese RA PreventIon of structural Damage (J-RAPID; ClininalTrials.gov, NCT00791999) for CZP, and the GO-MONO [17] and GO-FORTH [18] for GLM. Although the study period of these clinical trials lasted more than 1 year, including extension studies for CZP and GLM, our study evaluated data only for 54 weeks of the RISING study and 52 weeks for the other four clinical trials. The measurement of serum KL-6 levels was originally scheduled in RISING, HIKARI, and J-RAPID, and the protocols and informed consent forms of GO-MONO and GO-FORTH were amended during these clinical trials to measure serum KL-6.

RISING study

The first clinical trial of biological DMARDs that included serum KL-6 as a laboratory test was RISING [15]. Electronic Supplementary Material (ESM) Fig. S1 shows the design and ESM Table S1 shows the baseline characteristics of the patients enrolled in RISING. In this trial, established RA patients with mean disease duration of 8.2 years received IFX for 54 weeks with concomitant stable doses of MTX. After a screening period, 327 patients entered the open-label period (3 mg/kg at weeks 0, 2, and 6) and 307 patients proceeded to the double-blind (DB) trial period. These patients were randomly allocated to 3, 6, or 10 mg/kg IFX groups and received an infusion of IFX every 8 weeks through to week 54. The percentage of patients with elevated serum KL-6 levels higher than 500 U/ml at baseline was 3.1 %.

HIKARI and J-RAPID

Two clinical trials for CZP have been implemented in Japan—HIKARI (phase III) and J-RAPID (phase II/III). In HIKARI, 230 RA patients who had an inadequate response to or who were intolerant of MTX were DB randomly assigned either to placebo or CZP without MTX for 24 weeks, followed by an open extension period until approval of the drug (ESM Fig. S2-A). In J-RAPID, 316 RA patients who had inadequate response to treatment with MTX were DB randomly allocated either to the placebo or to one of three dosage groups of CZP with concomitant MTX at stable dosages for 24 weeks, followed by an open extension period until approval of the drug (ESM Fig. S2-B). Both trials allowed for early escape (EE) at week 16 if a patient did not meet ACR20 response criteria at both weeks 12 and 14. Demographic characteristics of the enrolled patients to these trials were similar, with a mean disease duration of about 6 years (ESM Table S2). The percentage of patients with IP in HIKARI was 12.2 % and in J-RAPID 2.2 %. The percentage of patients with elevated serum KL-6 levels of $\geq$500 U/ml at baseline was 8.8–11.2 % in HIKARI and 2.4–6.1 % in J-RAPID (ESM Table S3).

GO-MONO and GO-FORTH

Serum KL-6 levels were evaluated in two randomized controlled trials of GLM implemented in Japan—GO-MONO [17] and GO-FORTH [18]. Patients who participated in either of these studies and who gave consent for measurements of serum KL-6 level were enrolled in our study. In GO-MONO, 308 RA patients who had an inadequate response to DMARDs were DB randomly assigned to placebo, GLM 50 mg, or GLM 100 mg monotherapy for 16 weeks, followed by an open extension period until week 116 (ESM Fig. S3-A). In GO-FORTH, 261 RA patients who had an inadequate response to MTX were DB randomly assigned to placebo, GLM 50 mg, or GLM 100 mg with concomitant MTX at stable dosages for 24 weeks, followed by an open extension period until week 152 (ESM Fig. S3-B). Baseline characteristics of the enrolled patients are summarized in Table ESM S4. The mean disease duration of the enrolled patients was about 9 years and patients with IP were not eligible for either study. At baseline for GO-MONO, 3.8 % of the patients in the GLM 50 mg group, 0 % in the GLM 100 mg group, and 1.3 % in the placebo group had KL-6 levels of >500 U/ml. In GO-FORTH, 2.9 % of the patients in the MTX + GLM 50 mg group, 0 % in the MTX + GLM 100 mg group, and 4.2 % in the MTX + placebo group had KL-6 levels of >500 U/ml (ESM Table S5).

Data collection

The chairperson (M.H.) and the committee members (A.T., T.A., M.D., S.H., H.N., and Y.S.) reviewed the data on elevations of serum KL-6 levels in the five Japanese clinical trials. M.H. and A.T. requested that the pharmaceutical companies Mitsubishi Tanabe Pharma Corporation, Otsuka Pharmaceutical, UCB Japan, and Janssen Pharmaceutical provide data in a systematic and predetermined format. The data were analyzed by the committee only; the pharmaceutical companies were not involved in data analysis. The committee did not have direct access to the database of the clinical trials. The final version of this report was reviewed by the pharmaceutical companies to enable data validation.

Measurement of serum KL-6 levels

Serum KL-6 levels were centrally measured in each clinical trial. In the RISING study, serum KL-6 levels were measured at weeks 0, 2, and then every 4 weeks until week 54. In HIKARI and J-RAPID, serum KL-6 levels were measured at weeks 0 and 1, every other week (EOW) from weeks 2 to 16, and at weeks 20 and 24 during the double-blind trial period, and every 4 weeks from weeks 28 to 52. Serum KL-6 levels were retrospectively measured at weeks 0, 12, 24, 36, and 52 for GO-MONO and GO-FORTH using stored serum samples. Serum KL-6 levels were available for 250 and 212 patients in GO-MONO and GO-FORTH, respectively. Serum KL-6 levels were also measured after week 52 in the clinical trials for CZP and GLM, but these data were not analyzed for our study. Serum KL-6 levels were measured using the Picolumi KL-6 kit (Eidia Co., Tokyo, Japan) in all five clinical trials.

Definition of elevated/reduced serum KL-6 levels

Criteria A, B, and C for the elevation of serum KL-6 levels were developed by the committee and are shown in Table 1. We defined three criteria based on the serum KL-6 value at the initiation of TNF inhibitor therapy and the maximum value thereafter because some patients with RA have elevated serum KL-6 levels due to concurrent pulmonary diseases at baseline. In this study, our primary focus was criterion B. We also defined criterion R for the significant reduction of serum KL-6 levels in RA patients after achieving criterion B (Table 1).

Association of elevated serum KL-6 level with pulmonary events

We analyzed the association of elevated serum KL-6 levels with pulmonary events through week 54 for IFX and week 52 for CZP and GLM. Pulmonary events of this study were defined using preferred terms of the MedDRA ver 12.0 including PCP (10064108), interstitial lung disease (10022611), pulmonary fibrosis (10037383), and pulmonary interstitial emphysema syndrome (10037415). In this study, we used the diagnosis of pulmonary events that were made during the clinical trials by the original investigators. Newly diagnosed or exacerbated pulmonary events between 4 weeks before and 4 weeks after the first elevation of serum KL-6 levels meeting criterion B were counted.

Statistical analysis

Percentages and incidence rates were calculated per 100 patient-years (PY) for patients who met the criteria for elevated serum KL-6 levels. Denominators were: 327 patients who received open-label treatment in RISING; full-analysis-set patients for whom data on KL-6 were available in HIKARI (230 patients) and J-RAPID (316 patients); patients who gave informed consent to serum KL-6 level measurements and for whom available data were available in GO-MONO (250 patients) and GO-FORTH (212 patients).

Because RISING did not have a placebo group and three different doses of IFX were compared for 54 weeks, the primary and secondary endpoints of our study for RISING were percentage and incidence rates per 100 PY of patients who met criterion B by week 54, respectively. Taking the time points when treatments were changed for open extension periods in HIKARI, J-RAPID, GO-MONO, and GO-FORTH into account (ESM Figs. S2, S3), we defined the primary endpoints as percentages of patients who met criterion B by week 28 for HIKARI and J-RAPID, by week 16 for GO-MONO, and by week 24 for GO-FORTH. Secondary endpoints for these four trials were percentages of patients who met criteria A and C and incidence rates per 100 PY of patients who met criteria A, B, and C by the same time points given above, and percentages and incidence rates per 100 PY of patients who met criteria A, B, and C by week 52 in each trial. Percentages among treatment groups were compared using the Fisher's exact probability test for the primary endpoints, but statistical comparisons were not calculated for secondary endpoints. In clinical trials comparing different dosage groups, the TNF inhibitor groups combined were first compared with the placebo group. If a significant difference was observed, each dosage group was then compared with the placebo group. We took these measures to avoid type I errors derived from multiple comparisons.

Ethics

The study protocols of the five clinical trials were approved by the local institutional review board of each study institution and were carried out in accordance with the Helsinki Declaration and Good Clinical Practice. In GO-MONO and GO-FORTH, patients provided additional informed consent after amendment of the study protocols to measure serum KL-6 levels using stored serum samples.

Table 1 Criteria for the elevation or reduction of serum KL-6 levels

Criteria	Definition
A	≥500 U/ml and ≥1.25-fold higher than baseline value
B	≥500 U/ml and ≥1.5-fold higher than baseline value
C	≥1,000 U/ml and ≥3-fold higher than baseline value
R	Decrease in serum KL-6 levels to <500 U/ml or less than [baseline + 0.5 × (maximum baseline)] after achieving criterion B and reaching the maximum level of a patient

KL-6 Krebs von den Lungen-6 antigen

Criteria A, B, and C are for the elevation of serum KL-6 levels, and criterion R is for the reduction of serum KL-6 levels after achieving criterion B and reaching the maximum level of a patient

Results

Elevation of serum KL-6 levels in RISING

Among the 327 patients who received open-label treatment with IFX, the percentage (incidence rate/100 PY) of patients by week 54 who met criterion A was 18.7 % (20.0/100 PY), criterion B 15.6 % (16.7/100 PY), and criterion C 1.5 % (1.6/100 PY) (Table 2). The percentages of patients meeting all three criteria in the 3 mg/kg group were not significantly different from those in the 6 or 10 mg/kg groups.

Table 2 Percentage and incidence rate/100 PY of patients meeting the criteria for elevated serum KL-6 levels at least one time by week 54 in RISING

Treatment group	Number of patients	Percentage and incidence rate		
		Criterion A	Criterion B	Criterion C
IFX (3 mg/kg)	99	16.2 % (16.6/100 PY)	14.1 % (14.5/100 PY)	1.0 % (1.0/100 PY)
IFX (6 mg/kg)	104	21.2 % (21.6/100 PY)	15.4 % (15.7/100 PY)	1.0 % (0.98/100 PY)
IFX (10 mg/kg)	104	18.3 % (18.4/100 PY)	16.3 % (16.5/100 PY)	2.9 % (2.9/100 PY)
All patients	327	18.7 % (20.0/100 PY)	15.6 % (16.7/100 PY)	1.5 % (1.6/100 PY)

Criteria A, B, and C for elevation of serum KL-6 levels are defined in Table 1. Among 327 patients who received open-label treatment with IFX (3 mg/kg), 20 patients did not enter the double-blind (DB) period. No significant difference exists in percentages of the patients meeting the three criteria in the 3 mg/kg group compared to the 6 or 10 mg/kg groups by the Fisher's exact probability test. Lengths of exposure were 96.4 PY for the 3 mg/kg IFX group, 101.7 PY for 6 mg/kg IFX group, 103.2 PY for 10 mg/kg IFX group, and 304.6 PY for all patients

IFX infliximab, *PY* patient-year

We analyzed the association between elevated serum KL-6 levels and the predefined pulmonary events described in "Patients and methods". Of the 51 cases meeting criterion B by week 54, three pulmonary events in three patients were reported (ESM Table S6). The serum KL-6 level of a suspected case of PCP at week 12 (withdrawn from the trial before entering the DB period) increased from 269 (week 0) to 996 U/ml (week 14), that of a patient who developed IP at week 6 (withdrawn from the trial before entering the DB period) increased from 468 (week 0) to 935 U/ml (week 6), and that of a patient developing IP at week 50 increased from 205 (week 0) to 1,470 U/ml (week 50). The remaining 48 patients did not develop any of the predefined pulmonary events, and we could not identify other specific reasons, including malignancy, for the elevated KL-6 levels in these patients.

Changes in serum KL-6 levels over time in RA patients meeting criterion B ($n = 51$) are shown in Fig. 1. In 29 (60.4 %) of the 48 RA patients who met criterion B without developing a predefined pulmonary event, serum KL-6 levels spontaneously decreased to meet criterion R by week 54. Of these 48 RA patients, 33 had serum KL-6 data available after reaching their maximum level of whom 29 (87.9 %) met criterion R by week 54.

Elevation of serum KL-6 levels in HIKARI

In HIKARI, patients who entered EE received 200 mg of CZP EOW on and after week 16, while treatments of patients who did not enter EE were changed at week 28 (ESM Fig. S2-A). We therefore performed on-drug analysis for weeks 0–28: the exposure period of patients who entered EE at week 16 included only the first 16 weeks in their originally allocated treatment group. The exposure period of patients who did not enter EE was 28 weeks or until withdrawal from the trial, whichever came first. Between weeks 0 and 28, 16 (13.8 %) of the patients who received CZP 200 mg without MTX satisfied criterion A and 9 (7.8 %) satisfied criterion B, while 4 (3.5 %) and 0 % of patients who received placebo without MTX met criteria A and B, respectively ($p = 0.009$ for criterion A; $p = 0.003$ for criterion B vs. placebo group by the Fisher's exact probability test) (Table 3). By week 52, of the 219 patients, 12.8 % (19.3/100 PY) met criterion A, 9.2 % (13.8/100 PY) met criterion B, and 1.4 % (2.1/100 PY) met criterion C. For this 52-week analysis, the exposure period of patients who were initially assigned to the placebo group included only the period of time they received CZP, that of patients who were assigned to the CZP 200 mg group was counted from weeks 0 to 52, and that of patients who were withdrawn from the clinical trial before week 52 included only the period before withdrawal.

We analyzed the association between elevated serum KL-6 levels and the occurrence of the defined pulmonary events described in "Patients and methods". One case of IP and two cases of PCP were reported among the 21 cases meeting criterion B by week 52. The serum KL-6 levels of the patient who developed IP at week 50 (CZP 200 mg group) increased from 428 (week 0) to 663 U/ml (week 52), those of the patient who developed PCP at week 6 (CZP 200 mg group) increased from 945 (week 0) to 3,610 U/ml (week 6), and those of the patient who developed PCP at week 24 (placebo group, but receiving CZP 200 mg at the development of PCP) increased from 383 (week 0) to 1,600 U/ml (week 30). The remaining 18 patients did not develop the predefined pulmonary events nor could we identify other specific reasons, including malignancy, for the observed elevation in KL-6 levels in these patients.

Changes in serum KL-6 levels in 6 patients in the placebo group and 15 in the CZP 200 mg group meeting criterion B are shown in Fig. 2. All patients from the placebo group met criterion B after their treatments were changed to 200 mg of CZP. In 7 (38.9 %) of the 18 RA patients who met criterion B without developing any of the

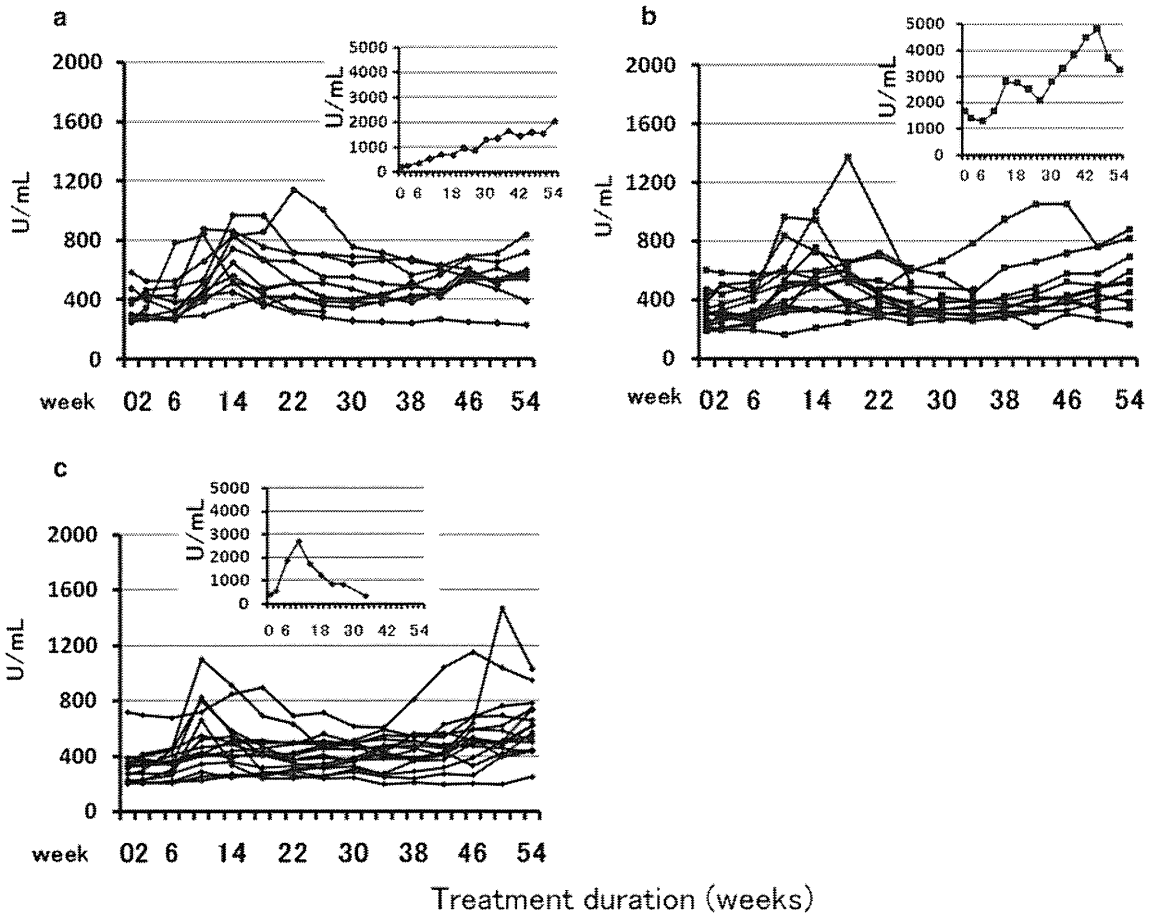

Fig. 1 Changes in serum Krebs von den Lungen-6 (KL-6) antigen levels over time in 51 rheumatoid arthritis (RA) patients who met criterion B at least one time by week 54 in the RISING study. Data from the infliximab (IFX) 3 mg/kg group ($n = 14$) and from patients who did not enter the double-blind (DB) period ($n = 4$) (**a**), data from the IFX 6 mg/kg group ($n = 16$) (**b**), and data from the IFX 10 mg/kg group ($n = 17$) (**c**) are shown separately. Data from patients whose maximum serum KL-6 level reached >2,000 U/ml are shown in the *insets* of the figures. For definition of criterion B, see Table 1

Table 3 Percentage and incidence rate/100 PY of patients who met the criteria for elevated serum KL-6 levels at least one time by week 28 in HIKARI

Treatment group	Number of patients[a]	Percentage and incidence rate		
		Criterion A	Criterion B	Criterion C
CZP (200 mg)	116	13.8 % (29.9/100 PY)*	7.8 % (16.8/100 PY)**	1.7 % (3.7/100 PY)
Placebo	114	3.5 % (10.6/100 PY)	0 % (0.0/100 PY)	0 % (0.0/100 PY)

Criteria A, B, and C for elevation of serum KL-6 levels are defined in Table 1. The exposure period of patients who entered early escape (EE) at week 16 was considered to be 16 weeks. The exposure period of patients who did not enter EE was considered to be 28 weeks or until withdrawal from the trial. Lengths of exposure were 53.5 PY for the CZP 200 mg group and 37.8 PY for the placebo group

CZP certolizumab pegol

Significance: * $p = 0.009$, ** $p = 0.003$ (CZP vs. placebo groups, by the Fisher's exact probability test)

[a] All patients assigned to each group with available data for serum KL-6 levels were evaluated

predefined pulmonary events, serum KL-6 levels spontaneously decreased to meet criterion R by week 52. Of these 18 RA patients, 14 had serum KL-6 data available after reaching their maximum levels of whom 7 (50.0 %) met criterion R by week 52.

Elevation of serum KL-6 levels in J-RAPID

Patients in J-RAPID who entered EE received 200 mg of CZP EOW with MTX on and after week 16, while treatments of patients who did not enter EE were changed at

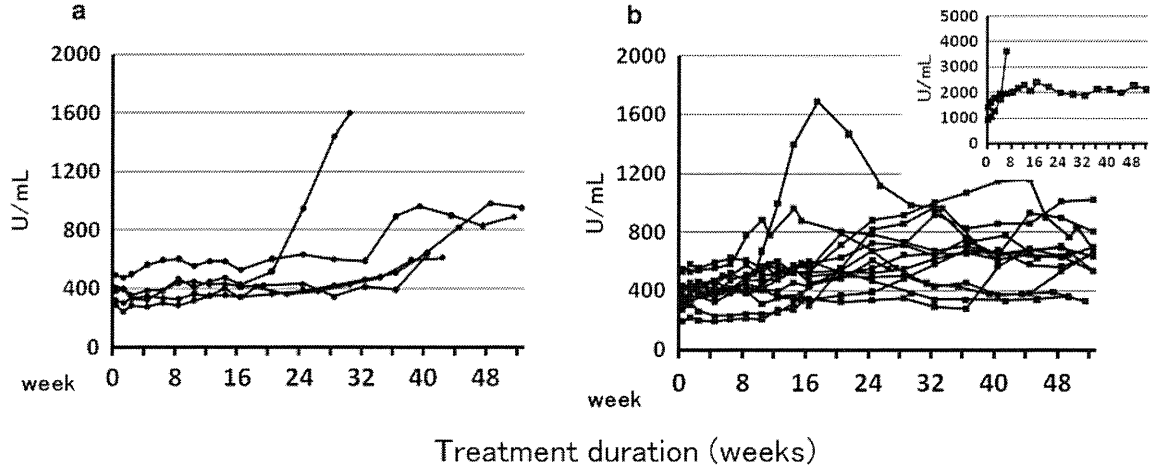

Fig. 2 Changes in serum KL-6 levels over time in 21 RA patients given certolizumab pegol (CZP) who met criterion B at least one time by week 52 in the HIKARI study. Data from the placebo group ($n = 6$) (**a**) and the CZP 200 mg group ($n = 15$) (**b**) are shown separately. The treatment for each patient was changed as described in ESM Fig. S2-A. Data from patients whose maximum serum KL-6 level surpassed 2,000 U/ml are shown in the *insets* of the figures

Table 4 Percentage and incidence rate/100 PY of patients who met the criteria for elevated serum KL-6 levels at least one time by week 28 in J-RAPID

Treatment group[a]	Number of patients[b]	Percentage and incidence rate		
		Criterion A	Criterion B	Criterion C
CZP (100 mg)	72	11.1 % (24.1/100 PY)	5.6 % (12.0/100 PY)	2.8 % (6.0/100 PY)
CZP (200 mg)	82	12.2 % (25.5/100 PY)	9.8 % (20.4/100 PY)	2.4 % (5.1/100 PY)
CZP (400 mg)	85	9.4 % (20.0/100 PY)	9.4 % (20.0/100 PY)	2.4 % (5.0/100 PY)
CZP (combined)	239	10.9 % (23.1/100 PY)	8.4 % (17.8/100 PY)	2.5 % (5.3/100 PY)
Placebo	77	6.5 % (18.0/100 PY)	3.9 % (10.8/100 PY)	0 % (0.0/100 PY)

Criteria A, B, and C for elevation of serum KL-6 levels are described in Table 1. The exposure period of patients who entered EE at week 16 was considered to be 16 weeks. The exposure period of patients who did not enter EE was taken to be 28 weeks or until withdrawal from the trial. Lengths of exposure were 33.2 PY for the MTX + CZP 100 mg group, 39.3 PY for the MTX + CZP 200 mg group, 39.9 PY for the MTX + CZP 400 mg group, and 27.7 PY for the MTX + placebo group. Percentages of the patients meeting the three criteria in the CZP groups combined did not differ significantly from the placebo group by the Fisher's exact probability test

MTX methotrexate

[a] All patients received placebo, CZP 100, 200, or 400 mg with concomitant MTX. CZP (combined) refers to the total of all CZP treatment group patients

[b] All patients who were assigned to each group with available data for serum KL-6 level were evaluated

week 28, the same as in HIKARI (ESM Fig. S2-B). We therefore performed on-drug analysis for weeks 0–28 as described for HIKARI. Between weeks 0 and 28, 4 (5.6 %) patients from the MTX + CZP 100 mg group, 8 (9.8 %) from the MTX + CZP 200 mg group, 8 (9.4 %) from the MTX + CZP 400 mg group, and 20 (8.4 %) from the MTX + CZP groups combined met criterion B, while 3 (3.9 %) patients from MTX + placebo group met criterion B (Table 4). No significant difference was found between the CZP groups combined and the placebo group. By week 52, of the 309 patients, 12.0 % (15.5/100 PY) met criterion A, 9.7 % (12.6/100 PY) met criterion B, and 2.6 % (3.4/100 PY) met criterion C. For this 52-week analysis, the exposure periods were the same as those described for HIKARI.

We analyzed the association between elevated serum KL-6 levels and the pulmonary events defined in "Patients and methods". Among the 32 cases meeting criterion B by week 52, no patients developed any of the predefined pulmonary events. We could not identify any other specific reasons, including malignancy, for the elevation of KL-6 serum levels in these 32 patients.

Changes in serum KL-6 levels in these 32 patients in the MTX + placebo (5 patients), the MTX + CZP 100 mg group (8), the MTX + CZP 200 mg group (9), and in the MTX + CZP 400 mg group (10) meeting criterion B are

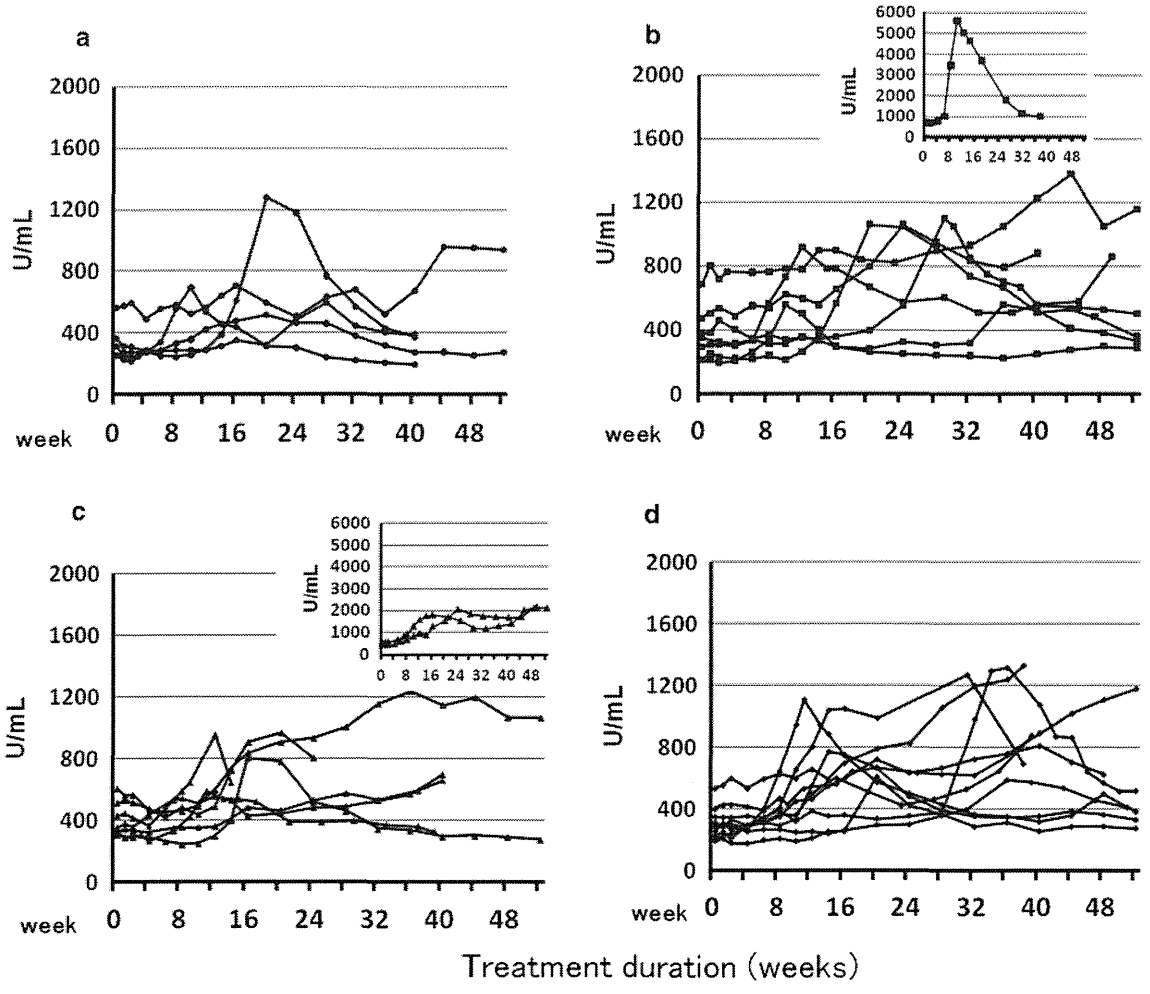

Fig. 3 Changes in serum KL-6 levels over time in 32 RA patients given CZP who met criterion B at least one time by week 52 in the J-RAPID study. Data from the methotrexate (MTX) + placebo group ($n = 5$) (**a**), MTX + CZP 100 mg group ($n = 8$) (**b**), MTX + CZP 200 mg group ($n = 9$) (**c**), and MTX + CZP 400 mg group ($n = 10$) (**d**) are shown separately. The treatment for each patient was changed as described in ESM Fig. S2-B. Data from patients whose maximum serum KL-6 level surpassed 2,000 U/ml are shown in the *insets* of the figures

depicted in Fig. 3. Three patients from the MTX + placebo group met criterion B while they were receiving placebo and 2 patients met criterion B after their treatments were changed to 200 mg of CZP. In 19 (59.4 %) of the 32 patients who met criterion B, serum KL-6 levels spontaneously decreased to meet criterion R by week 52. In these 32 RA patients, 27 had serum KL-6 data available after reaching their maximum level of whom 19 (70.4 %) met criterion R by week 52.

Elevation of serum KL-6 levels in GO-MONO

In GO-MONO, study blindness was maintained until week 16 and there was no EE. Patients from the placebo group started 50 mg GLM on and after week 16 (ESM Fig. S3). By week 16, 1 (1.3 %) patient in the GLM 50 mg group, 2 (2.2 %) patients in the GLM 100 mg group, 3 (1.8 %) patients in the GLM groups combined, and 1 (1.3 %) patient in the placebo group met criterion B (Table 5). No significant difference between the GLM groups combined and the placebo group was found. By week 52, of the 250 patients, 8.0 % (8.8/100 PY) met criterion A, 6.8 % (7.5/100 PY) met criterion B, and 0.8 % (0.9/100 PY) met criterion C. For this 52-week analysis, the exposure period of patients who were initially assigned to the placebo group was counted only for the period when they received GLM and the exposure period of patients who were assigned to the GLM groups was counted from weeks 0 to 52. The exposure period of patients who were withdrawn from the clinical trial before week 52 included only the period before withdrawal.

We analyzed the association between elevated serum KL-6 levels and the pulmonary events described in "Patients and methods". Among the 17 cases meeting

Table 5 Percentage and incidence rate/100 PY of patients who met the criteria for elevated serum KL-6 levels at least one time by week 16 in GO-MONO

Treatment group[a]	Number of patients[b]	Percentage and incidence rate		
		Criterion A	Criterion B	Criterion C
GLM (50 mg)	79	1.3 % (4.1/100 PY)	1.3 % (4.1/100 PY)	0.0 % (0.0/100 PY)
GLM (100 mg)	91	2.2 % (7.1/100 PY)	2.2 % (7.1/100 PY)	0.0 % (0.0/100 PY)
GLM (combined)	170	1.8 % (5.7/100 PY)	1.8 % (5.7/100 PY)	0.0 % (0.0/100 PY)
Placebo	80	1.3 % (4.1/100 PY)	1.3 % (4.1/100 PY)	0.0 % (0.0/100 PY)

Percentages of the patients meeting the three criteria in the GLM groups combined did not differ significantly from the placebo group by the Fisher's exact probability test. Criteria A, B, and C for elevation of serum KL-6 levels are described in Table 1. The exposure period of patients who were withdrawn from the trial before week 16 was counted only for the period until the withdrawal. Lengths of exposure were 24.5 PY for the GLM 50 mg group, 28.2 PY for the GLM 100 mg group, and 24.7 PY for the placebo group

GLM Golimumab

[a] GLM (combined) refers to the total of all GLM treatment group patients

[b] Number of patients who gave consent to measure serum KL-6 levels and had available data

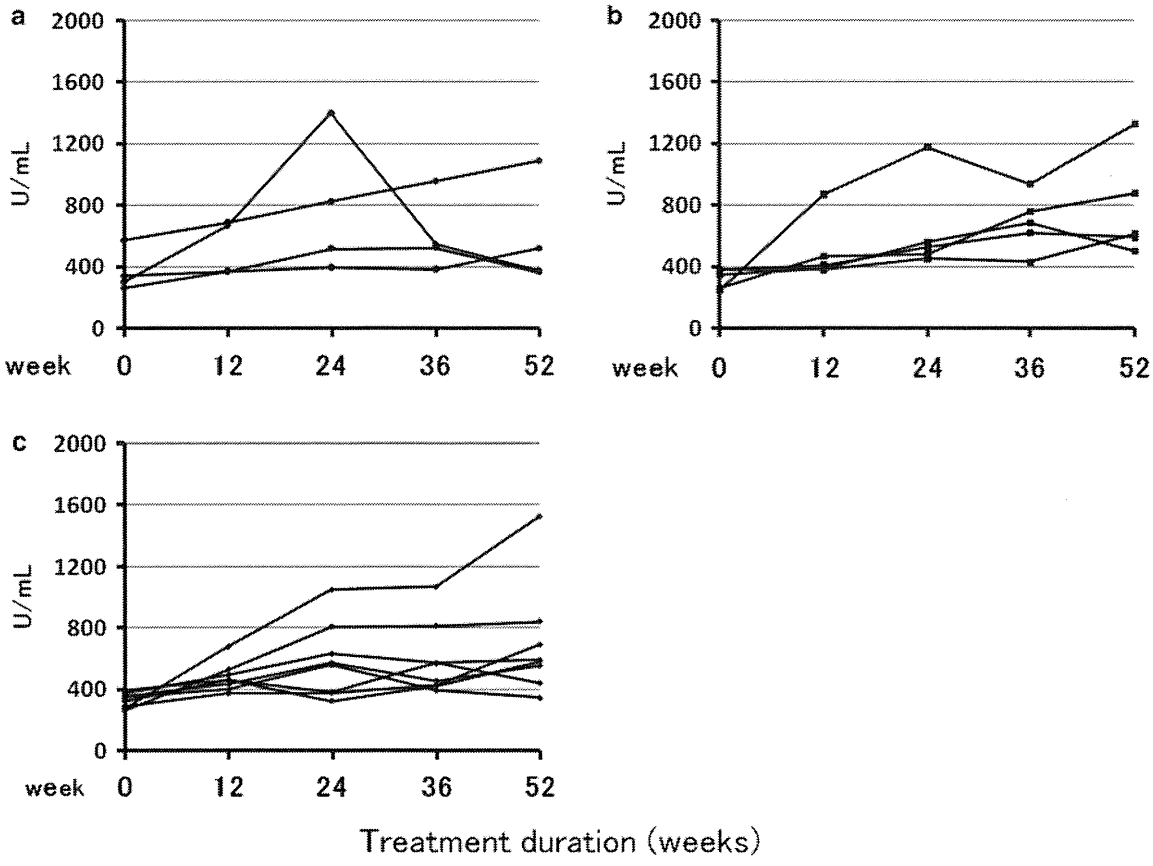

Fig. 4 Changes in serum KL-6 levels over time in 17 RA patients given golimumab (GLM) who met criterion B at least one time by week 52 in the GO-MONO study. Data from the placebo group ($n = 4$) (**a**), GLM 50 mg group ($n = 5$) (**b**), and GLM 100 mg group ($n = 8$) (**c**) are shown separately. The treatment for each patient was changed as described in ESM Fig. S3

criterion B by week 52, no patients developed the predefined pulmonary events, but one case of organizing pneumonia was reported by week 52. We could not identify other specific reasons, including malignancy, for the elevation of KL-6 serum levels in these 17 patients.

Changes in serum KL-6 levels over time in these 17 RA patients meeting criterion B in the placebo group (4 patients), the GLM 50 mg group (5), and the GLM 100 mg group (8) are shown in Fig. 4. One patient from the placebo group met criterion B when receiving placebo, while 3

patients met criterion B after their treatments were changed to 50 mg of GLM. Serum KL-6 levels spontaneously decreased to meet criterion R by week 52 in 6 (35.3 %) of the 17 RA patients. Of these 17 RA patients, 7 had serum KL-6 data available after reaching their maximum level of whom 6 (85.7 %) met criterion R by week 52.

Elevation of serum KL-6 levels in GO-FORTH

Patients in GO-FORTH who entered EE at week 16 from the MTX + placebo group received 50 mg of GLM with MTX and the MTX + GLM 50 mg group received 100 mg. All patients in the MTX + placebo group who did not enter EE received 50 mg of GLM at week 24 (ESM Fig. S3). We therefore performed on-drug analysis for weeks 0–24. The exposure period of patients who entered EE at week 16 included only the first 16 weeks in their originally allocated treatment group. The exposure period of patients who did not enter EE was 24 weeks or until withdrawal from the trial, whichever came first. Between weeks 0 and 24, 3 (4.4 %) patients from the MTX + GLM 50 mg group, 7 (9.7 %) patients from the MTX + GLM 100 mg group, and 10 (7.1 %) patients from MTX + GLM groups combined satisfied criterion B, while no patients from the MTX + placebo group met criterion B ($p = 0.017$ for GLM groups combined and $p = 0.013$ for GLM 100 mg group using the Fisher's exact probability test) (Table 6). By week 52, of the 212 patients, 9.4 % (10.9/100 PY) met criterion A, 9.0 % (10.4/100 PY) met criterion B, and 0 % (0/100 PY) met criterion C. For this 52-week analysis, the exposure periods were the same as those described for GO-MONO.

We analyzed the association between elevated serum KL-6 levels and pulmonary events as defined in "Patients and methods". Among the 19 cases meeting criterion B by week 52, no patients developed the predefined pulmonary events. We could not identify other specific reasons, including malignancy, for the elevation of KL-6 serum levels in these patients.

Changes in serum KL-6 levels over time in these 19 RA patients meeting criterion B in the MTX + placebo (6 patients), the MTX + GLM 50 mg group (5), and the MTX + GLM 100 mg group (8) are depicted in Fig. 5. All patients from the MTX + placebo group met criterion B after their treatments were changed to 50 mg of GLM with MTX. Serum KL-6 levels spontaneously decreased to meet criterion R by week 52 in ten (52.6 %) of these 19 RA patients. Of these 19 RA patients, 11 had serum KL-6 data available after reaching their maximum level of whom 10 (90.9 %) met criterion R by week 52.

Discussion

The major findings of our study are that: (1) the use of TNF inhibitors was significantly associated with elevated serum KL-6 levels compared to placebo in two of the four clinical trials studied; (2) 8.0–18.6 % of RA patients given TNF inhibitors met criterion A, 6.8–15.3 % met criterion B, and 0–2.6 % met criterion C by year 1; (3) 134 (95.7 %) of 140 patients who met criterion B did not have any other specific clinical reasons for the elevation of serum KL-6 levels and the serum marker spontaneously decreased in the majority of these patients.

While we have presented data for serum KL-6 levels during treatment with TNF inhibitors from five clinical trials in a similar manner in our attempt to compare these trials, it should be noted that the frequency of the

Table 6 Percentage and incidence rate/100 PY of patients who met the criteria for elevated serum KL-6 levels at least one time by week 24 in GO-FORTH

Treatment group[a]	Number of patients[b]	Percentage and incidence rate		
		Criterion A	Criterion B	Criterion C
GLM (50 mg)	68	4.4 % (9.8/100 PY)	4.4 % (9.8/100 PY)	0.0 % (0.0/100 PY)
GLM (100 mg)	72	9.7 % (20.9/100 PY)	9.7 % (20.9/100 PY)**	0.0 % (0.0/100 PY)
GLM (combined)	140	7.1 % (15.6/100 PY)	7.1 % (15.6/100 PY)*	0.0 % (0.0/100 PY)
Placebo	72	1.4 % (3.4/100 PY)	0.0 % (0.0/100 PY)	0.0 % (0.0/100 PY)

Criteria A, B, and C for elevation of serum KL-6 levels are defined in Table 1. The exposure period of patients who entered EE at week 16 was considered to be 16 weeks. The exposure period of patients who did not enter EE was considered to be 24 weeks or until withdrawal from the trial. Lengths of exposure were 30.7 PY for the MTX + GLM 50 mg group, 33.5 PY for the MTX + GLM 100 mg group, and 29.8 PY for the MTX + placebo group

Significance * $p = 0.017$ (the GLM groups combined vs. placebo group), ** $p = 0.013$ (the GLM 100 mg vs. placebo group) by the Fisher's exact probability test

[a] In GO-FORTH, patients received placebo, GLM 50 mg, or GLM 100 mg with concomitant MTX. GLM (combined) refers to the total of all GLM treatment group patients

[b] Number of patients who gave consent to measure serum KL-6 levels and for whom data were available

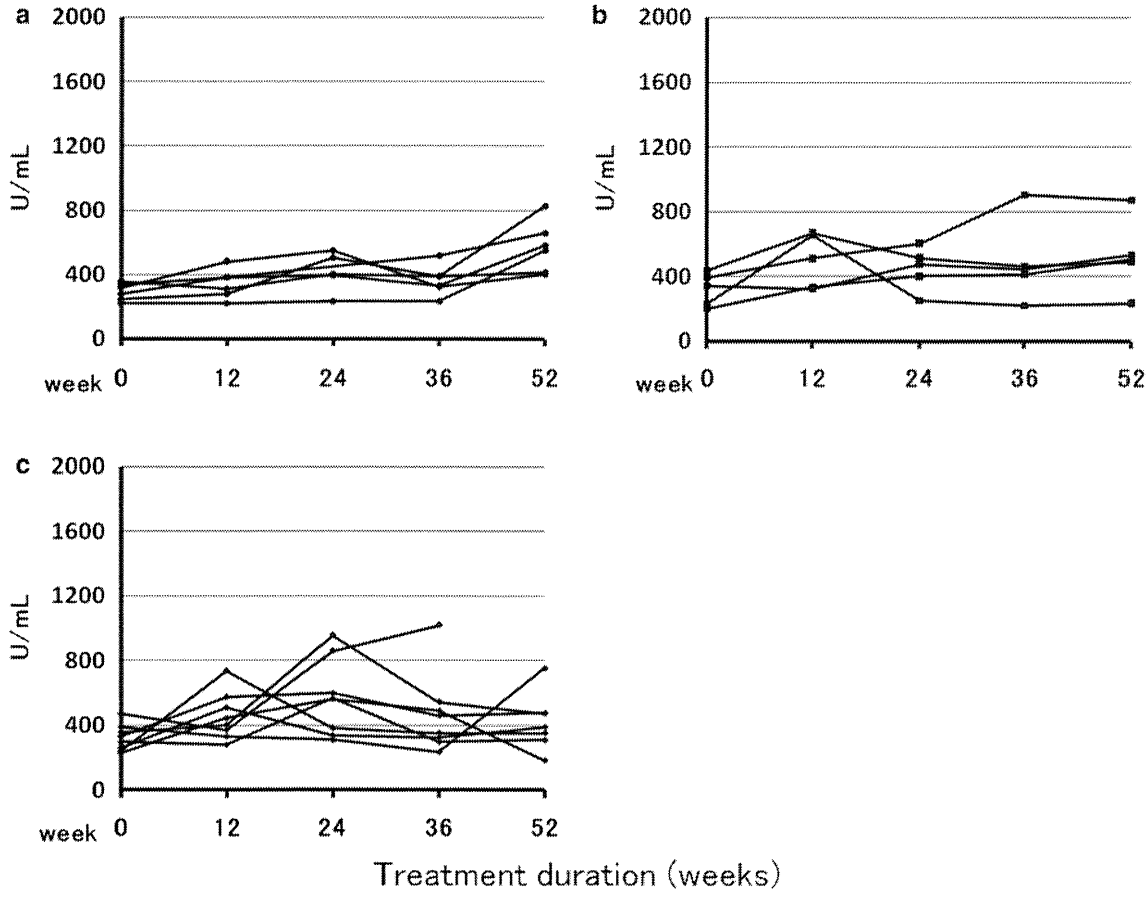

Fig. 5 Changes in serum KL-6 levels over time in 19 RA patients given GLM who met criterion B at least one time by week 52 in the GO-FORTH study are shown. Data from the MTX + placebo group ($n = 6$) (**a**), MTX + GLM 50 mg group ($n = 5$) (**b**), and MTX + GLM 100 mg group ($n = 8$) (**c**) are shown separately. The treatment for each patient was changed as described in ESM Fig. S3

measurement of serum KL-6 levels differed among these clinical trials and that the designs of the trials varied in terms of length of placebo-controlled and DB periods, EE, and treatment changes after the DB periods (ESM Figs. S1, S2, S3). The frequency of KL-6 measurement was highest in HIKARI and J-RAPID, followed by RISING, GO-MONO, and GO-FORTH. Because the spontaneous reduction of serum KL-6 levels was observed in all clinical trials, less frequent measurements may result in lower percentages of patients meeting the criteria for elevation of serum KL-6 levels. It should also be noted that the patient populations were different among the five clinical trials because of their mutually independent eligibility criteria. These differences should be considered when our findings are interpreted.

In HIKARI and J-RAPID, serum levels of pulmonary surfactant protein D (SP-D), another marker for interstitial lung disease [19], were retrospectively measured and visually compared with changes in serum KL-6 levels over time in some patients who met criterion B and had relatively high serum KL-6 levels. Both serum markers increased in parallel in about half of these patients (data not shown). Serum lactate dehydrogenase levels were also measured in these patients, but these did not correlate with serum KL-6 levels. These data indicate that the elevation of serum KL-6 levels in RA patients given TNF inhibitors was not a non-specific fluctuation, but may be associated with subclinical interstitial changes in the lung or that TNF may have a physiological role in regulatory pathways common to both serum markers.

In Japan, PCP is one of the most clinically important opportunistic infection in RA patients during treatment with TNF inhibitors [3, 4, 20]. Because serum KL-6 levels frequently increase in patients with PCP [10], the elevation of serum KL-6 levels in RA patients given TNF inhibitors may be explained by subclinical PCP. However, chest X-ray or thoracic computed tomography has not supported this hypothesis (data not shown). Serum levels of beta-D-glucan (BDG), a marker for PCP [21], were prospectively measured in HIKARI and J-RAPID. Of 21 patients who met criterion B by week 52 in HIKARI, an abnormal elevation of serum BDG levels ($\geq$11.0 pg/ml) was observed

in only two (9.5 %) patients (peak values 14.4 and 32.7 pg/ml, respectively). The patient with a peak value of 14.4 pg/ml developed PCP with the simultaneous elevation of serum KL-6 and BDG levels. In J-RAPID, of 32 patients who met criterion B by week 52, only 3 (9.4 %) patients (peak values 11.5, 12.3, and 18.1 pg/ml, respectively) showed abnormal, but modest, elevations of serum BDG levels that were observed in parallel with an elevation of serum KL-6 levels. These data and the favorable clinical courses of these patients indicate that the possibility of subclinical PCP was quite low in the majority of patients meeting criterion B.

Some important clinical questions arise from our findings. First, do we have to stop treatment with TNF inhibitors in RA patients with elevated serum KL-6 levels? The answer is no. We should search for reasons for the elevation, such as PCP, IP, and malignancy, as the first step, but when these adverse events are not identified, continuing treatment with TNF inhibitors under careful observation is a reasonable option for RA patients who have shown good response to the treatment. Second, is it worthwhile to monitor KL-6 every 4 weeks during treatment with TNF inhibitors? When we used criterion B to define the elevation of serum KL-6 levels, KL-6 had low positive predictive values for PCP and IP in RISING (5.9 %), HIKARI (14.3 %), and J-RAPID (0 %) and high negative predictive values (99–100 %) in all three trials. However, in these studies, there were only three PCP and three IP patients among those who met criterion B and three IP patients among those who did not met criterion B; there is, therefore, a possibility that more stringent criteria would have better predictive abilities for these adverse events. To clarify the usefulness of monitoring KL-6 serum levels during treatment with TNF inhibitors, a specifically designed clinical study is required. Therefore, we cannot provide a definite answer for the second question from our present analysis.

The potential contribution of concomitant MTX to the elevation of serum KL-6 levels in RA patients given TNF inhibitors should be mentioned. When we combined the MTX + placebo groups from J-RAPID and GO-FORTH, 3 (2.0 %) of 149 patients given MTX + placebo met criterion B by week 24 or 28 without associated pulmonary events. In our retrospective study [22], 5 (10.6 %) of 47 RA patients given MTX without biological DMARDs met criterion B, and 4 of these (8.5 %) did not have any clinical reasons for the elevation of serum KL-6 levels. These data indicate that we should consider a potential contribution of MTX to the elevation of serum KL-6 levels during treatment with TNF inhibitor + MTX.

The mechanisms of the elevation of serum KL-6 levels in RA patients given TNF inhibitors remain to be determined. Little is known about the molecular mechanisms of KL-6 expression and its transport mechanism through the alveolar–capillary barrier. Further studies are required to clarify the roles of TNF in these processes in both physiological and pathological conditions.

In summary, the transient elevation of serum KL-6 levels in patients meeting criterion B, without accompanying specific clinical events, was observed in 6.8–15.6 % of RA patients treated with TNF inhibitors by year 1 in five clinical trials. Continuing treatment with TNF inhibitors under careful observation is a clinically reasonable option when serum KL-6 levels rise.

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Conflict of interest The Ad Hoc Committee for Safety of Biological DMARDs of the Japan College of Rheumatology did not receive any financial support from industries and independently investigated and discussed the issues and prepared this manuscript. M.H. has received research grants from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, Eisai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical, and Pfizer and received consultant fees from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, and Janssen Pharmaceutical. T.A. has received research grants from Chugai Pharmaceutical and Takeda Pharmaceutical. H.K. has received honoraria from Abbott, Mitsubishi Tanabe Pharma Corporation, and Pfizer. Y.S. has received consultant fee from Abbott. T.K. has received consultancies, speaking fees and honoraria from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, Eisai Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical, and Pfizer, Otsuka Pharmaceutical. N.M. has received research grants from Abbott, Astellas Pharmaceutical Banyu Pharmaceutical, Chugai Pharmaceutical, Daiichi Sankyo Pharmaceutical, Eisai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical, and Teijin Pharmaceutical.

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ORIGINAL ARTICLE

A retrospective study of serum KL-6 levels during treatment with biological disease-modifying antirheumatic drugs in rheumatoid arthritis patients: a report from the Ad Hoc Committee for Safety of Biological DMARDs of the Japan College of Rheumatology

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Abstract
Objective We investigated associations between treatment with methotrexate (MTX) or biological disease-modifying antirheumatic drugs (DMARDs) and elevation of serum Krebs von den Lungen-6 (KL-6) levels in Japanese patients with rheumatoid arthritis (RA).
Methods Using a standardized form, data were collected retrospectively from medical records and analyzed descriptively.
Results Of a total of 198 RA patients with KL-6 serum levels measured at initiation of treatment (month 0) and two or more times by month 12, 27 (17.9 %) of 151 RA patients treated with biological DMARDs, including infliximab, etanercept, adalimumab, and tocilizumab (the biological DMARDs group), and 5 (10.6 %) of 47 patients treated without biological DMARDs but with MTX (MTX group), met criterion B (max. KL-6 $\geq$500 U/ml and >1.5-fold from baseline) by 12 months. The majority of patients ($n = 28$) meeting criterion B had no apparent interstitial lung disease or malignancy. Of these 28 patients, 21 had serum KL-6 levels available after reaching their maximum level, and 13 (61.9 %) of the 21 then met criterion R [decrease to less than 500 U/ml or to less than (baseline + 0.5 × (maximum − baseline))] by month 12.
Conclusion Serum KL-6 levels may increase during treatment with MTX or these biological DMARDs without significant clinical events.

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Keywords Rheumatoid arthritis · Biological disease-modifying antirheumatic drug · KL-6

Introduction

Six biological disease-modifying antirheumatic drugs (DMARDs), infliximab (IFX), etanercept (ETN), adalimumab (ADA), tocilizumab (TCZ), abatacept, and golimumab, have been approved in Japan. Today, these biological DMARDs are widely used for treatment of rheumatoid arthritis (RA). Pulmonary diseases with interstitial lesion, including rheumatoid lung, drug-induced pulmonary injury, or *Pneumocystis jirovecii* pneumonia (PCP), sometimes develop during treatment of RA with biological DMARDs [1–4]. Better prognosis for affected patients would be provided by prompt diagnosis of these diseases [5–8].

Krebs von den Lungen-6 (KL-6) is a circulating high-molecular-weight glycoprotein recently classified in humans as a cluster 9 mucin-1 (MUC1) [9]. The serum level of KL-6 was reported to be elevated in patients with idiopathic interstitial pneumonia (IIP), interstitial pneumonia (IP) associated with collagen diseases, IP associated with drug allergy, PCP, other interstitial lung diseases, and malignancy [10–15]. Measurement of serum KL-6 levels is an officially approved laboratory test in Japan, widely used as an adjunctive diagnostic or monitoring tool for patients with interstitial lung diseases and patients with RA treated with MTX or biological DMARDs.

In the Impact on Radiographic and clinical response of Infliximab therapy concomitant with methotrexate in rheumatoid arthritis patients by the trough Serum level in the dose-escalatING (RISING) study [16], abnormal elevation of serum KL-6 levels in RA patients treated with IFX with no pulmonary diseases was seen. Considering the prevalent use of serum KL-6 levels as a laboratory test for RA patients in Japan, the Japan College of Rheumatology (JCR) convened the Ad Hoc Committee for Safety of Biological DMARDs to investigate the abnormal elevation of serum KL-6 levels in RA patients during treatment with biological DMARDs. The committee implemented two studies, one using clinical trial data and one clinical practice data. Here, we report the results from a retrospective analysis of clinical practice data.

Patients and methods

Data source

Criteria for admission to this study included those patients (1) meeting the 1987 American College of Rheumatology criteria for RA [17], (2) ≥20 years old, (3) willing to provide informed consent, (4) starting treatment with biological DMARDs (IFX, ETN, ADA, and TCZ) or MTX, and (5) having serum KL-6 levels measured at start of treatment (month 0) and two or more times by month 12. Exclusion criteria included those patients (1) withdrawing consent to join the study, or (2) found to be unsuitable for the study at the discretion of the attending physician. Data, including age, gender, comorbidities, past history, disease duration, laboratory data [KL-6, surfactant protein-D (SP-D), beta-D-glucan, white blood cell counts, lymphocyte cell counts, lactate dehydrogenase, C-reactive protein, and erythrocyte sedimentation rate] at months 0, 6, and 12, number of tender and swollen joints at months 0, 6, and 12, the patients' global assessments at months 0, 6, and 12, and treatments during the 12 months, were collected from medical records using a standardized case report form. We also collected data on pulmonary events, including PCP, IP, and others, and any malignancies during the 12 months. Data on pulmonary events included diagnosis, date of diagnosis and prognosis of pulmonary events, and laboratory data, results of imaging analyses, and treatments at onset of pulmonary events.

Measurement of serum KL-6 levels and the criteria for increase and decrease

Serum KL-6 levels were measured using Picolumi KL-6 (Eidia Co., Ltd., Tokyo, Japan) or Lumipulse Presto KL-6 (Eidia Co., Ltd., Tokyo, Japan) by in-house laboratories or outsourced, depending on the institution. Baseline serum KL-6 levels were measured within 1 month from initiation of treatment (month 0). We defined elevation of serum KL-6 levels as follows: criterion A (max. KL-6 ≥500 U/ml and >1.25-fold from baseline), criterion B (max. KL-6 ≥ 500 U/ml and >1.5-fold from baseline), and criterion C (max. KL-6 ≥1000 U/ml and >3.0-fold from baseline). Reduction of serum KL-6 levels was defined as a decrease to less than 500 U/ml or to less than [baseline + 0.5 × (maximum − baseline)] after meeting criterion B and achieving the maximum level of an individual patient (criterion R).

Statistical analysis

In consideration of the retrospective nature of our study and unavoidable biases in selection of enrolled patients, we restricted the statistics to descriptive analysis. The chi-square test was used to compare categorical variables.

Ethics

The guidelines of the Helsinki Declaration and the ethics guidelines for epidemiological research in Japan were