of uveitis is one of the main differences between polyarticular and oligoarticular JIA (Table 2).[4] Uveitis is one of the most devastating complications of JIA, because it causes posterior synechia, band keratopathy and cataract, which can result in a poor visual prognosis.[22,23] Clinical risk factors for JIA-associated uveitis were also found to be associated with JIA-associated uveitis in Japanese (Table 2). In addition to these known risk factors, we found that HLA-A*02:06 was one of the risk factors. Using a two-locus analysis, HLA-A*02:06 and HLA-DRB1*09:01 were found to synergistically interact to increase susceptibility to JIA accompanied by uveitis. Because HLA-A*02:06 and HLA-DRB1*09:01 are not in linkage disequilibrium in Japanese,[24] two different genes in the HLA region might be responsible.

No patients with JIA accompanied by uveitis typed HLA-DRB1*13 positive in this study, although this allele has been reported to be significantly associated with such Caucasian patients.[8] There are several subtypes of HLA-DRB1*13; for example, DRB1*13:01 and DRB1*13:02, which vary in different ethnic groups. Because the majority of the DRB1*13 subtype in Japanese is DRB1*13:02, whereas it is DRB1*13:01 in Caucasians, the lack of association with DRB1*13 in our study might reflect these ethnic differences. In this regard, it should be noted that HLA-A*02:06 and HLA-DRB1*09:01 are relatively frequent in Japanese, but rare in European populations.

In this study, we demonstrated that JIA accompanied by uveitis was associated with HLA-A*02:06, which was not otherwise associated with JIA. This suggests that JIA accompanied by uveitis might be a specific clinical entity. It should be noted that HLA-A*02 (A2) is associated with susceptibility to anterior uveitis in adult patients[25] and JIA-associated uveitis in Caucasians.[14] In this regard, we should carefully follow-up the JIA patients carrying HLA-A*02:06 with respect to ocular complications.

We could not validate the association in this study. Although the genetic association should be validated in another cohort, the incidence of JIA and uveitis is too low to be validated in a single institution. Therefore, it is important to replicate the association between HLA-A*02:06/-DRB1*09:01 and JIA accompanied by uveitis in other cohorts including various ethnic groups.

In conclusion, we found an association of HLA-A*02:06 and possibly HLA-DRB1*09:01 with susceptibility to JIA accompanied by uveitis, which might be considered as a distinct clinical entity within JIA. Clinical subtypes of JIA may be classified by the presence of the specific HLA alleles, such as HLA-A*02:06 and DRB1*04:05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Original Article

National survey of childhood febrile illness cases with fever of unknown origin in Japan

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Abstract ***Background***: In Japan, an actual condition survey on childhood febrile diseases with fever of unknown origin has never been performed. We carried out a national survey on childhood febrile illnesses in order to collect useful information for the differential diagnosis.
Methods: A nationwide survey using questionnaires was performed on febrile illness cases with fever of unknown origin (FUO) experienced by 2843 pediatrics institutions with sick beds during a 5-year period before 2007. FUO was defined as a febrile illness of at least 2 weeks' duration with a temperature ≥38°C, and failure to establish a diagnosis in spite of intensive evaluation during seven days' hospitalization.
Results: Two hundred fifty-five of 2843 questionnaire-surveyed institutions had 960 FUO cases, of which 132 could not be diagnosed, and 828 could be diagnosed after detailed medical examinations. The diagnoses they clarified included infectious diseases in 190 cases (23%), rheumatic diseases in 448 cases (54%), neoplasms in 67 cases (8%), and others in 123 cases (15%).
Conclusion: Clarification of illnesses that ought to be differentiated in the diagnostic approach to an FUO case is essential for arriving at its definitive diagnosis by exclusion.

Key words child, febrile diseases, fever of unknown origin, final diagnosis, national survey.

We often experience cases with fever of unknown origin (FUO) in a clinical setting, yet research on the actual state of childhood febrile illnesses has rarely been done in our country, even though such research would be useful for the differential diagnosis of FUO. It is unclear in many aspects what diagnoses are made for FUO cases and how their differential diagnosis is made. Making a definite diagnosis of an FUO case is considered important for determination of therapeutic indication for an FUO case that really needs treatment. Therefore, we made a nationwide survey on childhood febrile illnesses on this occasion in order to acquire useful information for the differential diagnosis of FUO.

Methods

Survey institutions were 2843 nationwide children's institutions with sick beds. They were asked to answer primary and secondary retrospective inquiries about FUO cases they had experienced during a 5-year period before 2007. FUO was defined as a febrile illness of at least 2 weeks' duration with a temperature ≥38°C and failure to establish a diagnosis in spite of evaluation during seven days' hospitalization.

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In the primary survey, the number of FUO cases, final diagnosis, sex, and age were investigated. The secondary survey was made to investigate in detail the symptoms and signs as well as differential diagnostic approaches taken in the cases that had been reported to have final diagnoses in the primary survey.

This study protocol was approved by the Ethics Committee of Yokohama City University Hospital (approval no. 042, approval date: 27 July 2007).

Results

Data of the primary and secondary surveys

In the primary survey, of 2843 institutions to which questionnaires were sent, 1071 (37.7%) returned the questionnaire sheets. Valid answers were acquired from 1045 institutions, excluding 26 where pediatrics departments were closed. A total of 255 institutions experienced 960 applicable cases (Fig. 1), of which 132 could not be diagnosed, and 828 were diagnosed after detailed examinations: infectious diseases in 190 (23%), rheumatic diseases in 448 (58%), neoplasms in 67 (8%), and others in 123 (15%) (Fig. 2).

In the secondary survey, we made a more detailed investigation on 828 cases that had been reported in the primary survey with their established diagnoses. We sent questionnaire sheets to 230 institutions, 146 of which returned valid answered sheets. Eighteen institutions replied but their data were invalid for

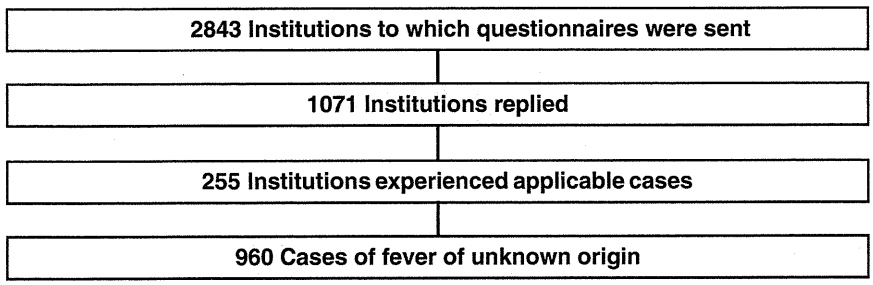

Fig. 1 Enrollment of primary survey.

analysis because their pediatrics departments were closed in some and case records that had been returned in the primary survey were incomplete in others. Of the above 146 institutions that answered properly, 127 reported 328 applicable cases (Fig. 3). These 127 institutions included 53 special hospitals, 60 municipal hospitals, and 14 non-specified facilities. Among the above 328 cases, only 185 met the above definitions.

Patients' backgrounds

A total of 101 patients were boys, and the male/female ratio was 1.2. Symptoms appeared at the age of 2 months to 18 years (mean, 7 years and 0 months) and diagnoses were made at the age of 2 months to 22 years (mean, 7 years and 3 months). Time from fever onset to diagnosis was 86.1 days on average. Diagnosis was established after close examinations in 153 out of 185 cases.

Classification of illnesses

There were 29 cases (15.7%) of infectious diseases, 108 (58.4%) of rheumatic diseases, 14 (7.6%) of neoplasms, and 34 (18.4%) of other diseases.

1 Infectious diseases (Fig. 4)
Cat scratch disease was most frequent in 10 cases, followed by seven cases of infectious diseases affected by viruses such as Epstein–Barr virus, coxsackie virus, adenovirus and others. Next

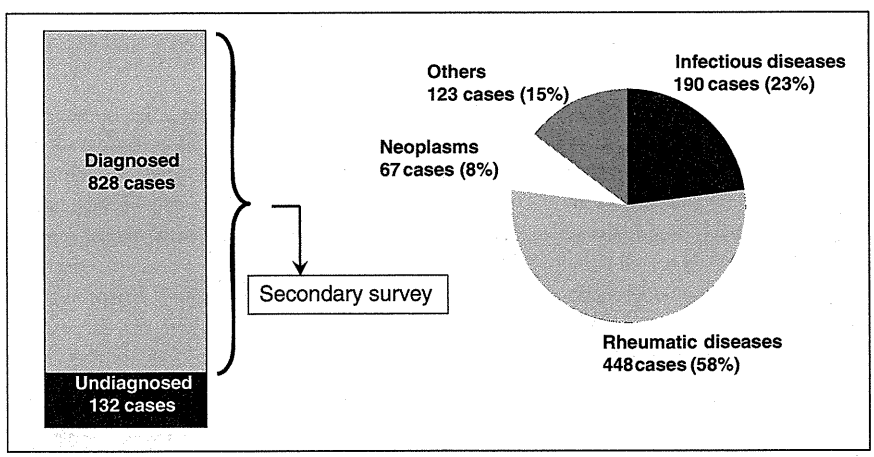

Fig. 2 Result of primary survey.

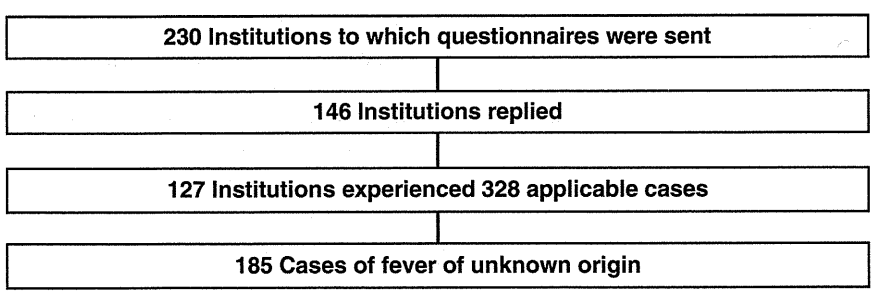

Fig. 3 Enrollment of secondary survey.

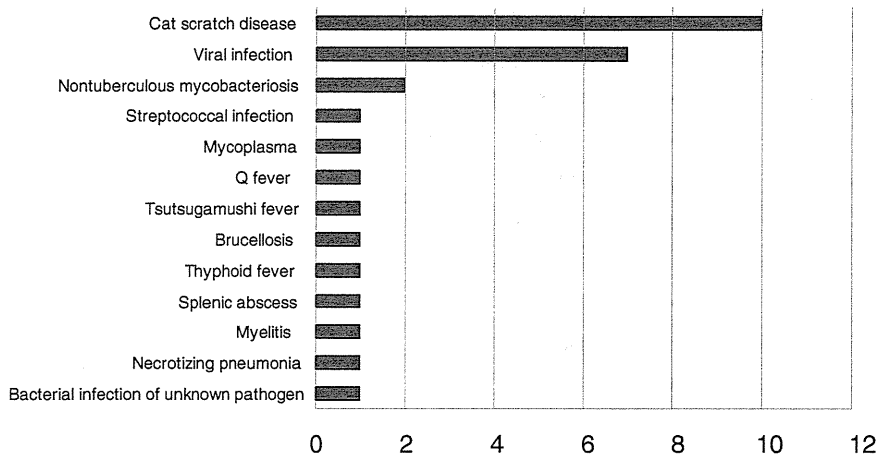

Fig. 4 Classification of infectious diseases.

came two cases of non-tuberculous mycobacteriosis. Rare cases of Q fever, tsutsugamushi fever, brucellosis, typhoid fever, and splenic abscess were included.

2 Rheumatic diseases (Fig. 5)

The most frequent illness was systemic-onset juvenile idiopathic arthritis, composing about 60% (68 cases). Others were nine cases of Takayasu's arteritis, eight cases of inflammatory bowel disease, and four cases of systemic lupus erythematosus.

3 Neoplasms

This category included five cases of Langerhans-cell histiocytosis, four cases of acute lymphocytic leukemia, two cases of

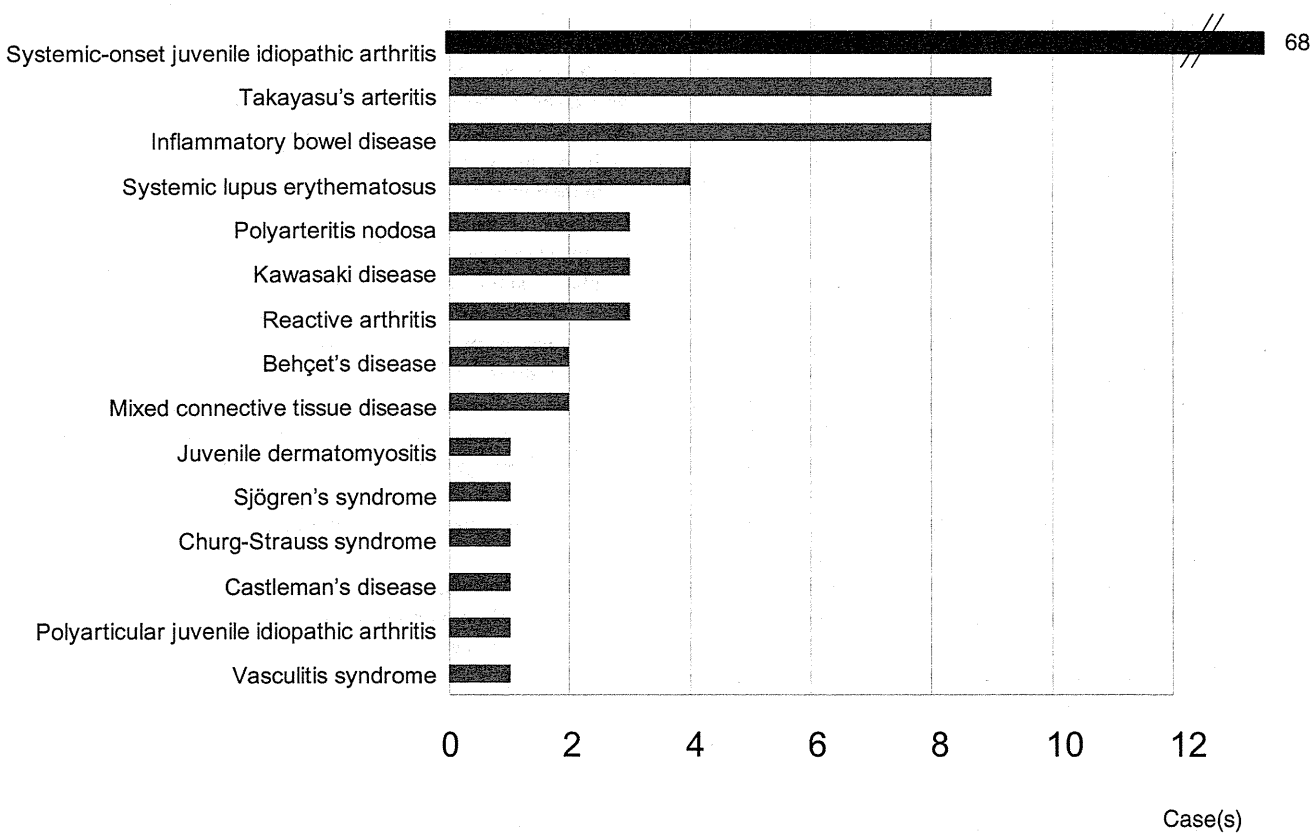

Fig. 5 Classification of rheumatic diseases.

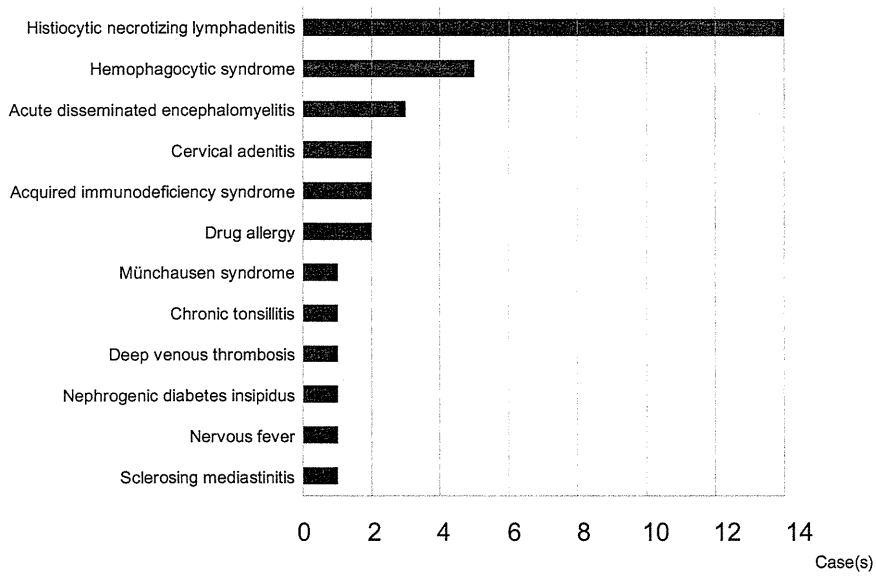

Fig. 6 Classification of other diseases.

malignant lymphoma, two cases of neuroblastoma, and one myofibromatosis case.

4 Others (Fig. 6)

Thirty-four other cases included 14 cases of histiocytic necrotizing lymphadenitis, which was most frequent, five cases of hemophagocytic syndrome, and three cases of acute disseminated encephalomyelitis.

Discussion

We surveyed the reality of cases with FUO that developed during 2003–2007 in order to study what illnesses were differentiated and to utilize it for the diagnostic approach of new FUO cases.

FUO was defined by Petersdorf *et al.* for the first time in 1961 as a febrile illness of at least three weeks' duration, with a temperature ≥38.3°C and failure to establish a diagnosis in spite of 1-week intensive inpatient evaluation.[1] The body temperature of 38.3°C was that measured in the oral cavity and could have been lower by 0.3–0.5 degrees if measured in the axilla. In 1968, Dechovitz *et al.* reported 155 cases of childhood FUO defined as a febrile illness of at least 2 weeks' duration with failure to identify a cause.[2]

In this study, in accordance with these reports, we defined a group of illnesses as a febrile illness with a temperature ≥38°C lasting for 2 weeks or longer and failure to establish a diagnosis in spite of evaluation during 1 weeks' hospitalization. After the report by Petersdorf *et al.*, several papers on FUO concerning pathogenetic classification in particular were published. However, papers on pediatric cases are very scarce, so that the present nationwide study performed in Japan is considered significant in this context.

Most of the papers roughly grouped the causes of FUO into infectious diseases, rheumatic diseases, neoplasms, others, and unknown in descending order of frequency (Pizzo *et al.* reported 100 prolonged fever cases in children: 52 were infectious, 20 collagen-inflammatory, six malignant, 10 miscellaneous, and 12 undiagnosed). Chantada *et al.* reported that 113 childhood FUO cases included 41 cases of infectious diseases, 15 of rheumatic diseases, 11 of neoplasms, and 22 of unknown cause.[3] As mentioned above, most studies concerning childhood FUO reported that infection was the most frequent cause of FUO.[4] In contrast, our present survey revealed that rheumatic diseases comprised the causes in 54%, which exceeded greatly 23% for infectious diseases. A similar trend was observed in a report concerning adults by Iikuni *et al.*[5]

In their report, among 79 adult FUO cases, 29.4% of them had rheumatic diseases, 28.8% infectious diseases, and 14.4% neoplasms, indicating a decrease in the rate of infectious diseases or neoplasms and an increase in that of rheumatic diseases as compared to previous reports. One of the reasons why rheumatic disease was the most common cause of FUO in the present study, as in the above report, was that it took a long time to make a diagnosis of illnesses associated with major conditions of systemic inflammation or vasculitis that had no specific markers. Systemic-onset juvenile idiopathic arthritis has no specific markers helpful for its diagnosis, so that symptoms such as skin rash and arthritis, are a determinant of reaching a diagnosis after all.

However, its diagnosis can be hard to make in the initial phase because of lack of pathognomonic symptoms or signs, including arthritis. This situation allowed the disease to fulfill the definition of FUO in many cases and the disease thus comprised the rheumatic disease group in around 60% in the present study.

Similarly, in an investigation of adult FUO by Goto *et al.*, adult-onset Still's disease, which simulated systemic-onset juvenile idiopathic arthritis in clinical conditions, comprised nearly 40% of the non-infectious inflammatory disease group, including rheumatic diseases.[6] Whereas it is still difficult to diagnose these

rheumatic diseases, the rate of the correct diagnosis of infectious diseases or neoplasms seems to be better than before. The spread of rapid diagnostic methods, the progress of antibody as well as culture examinations, and the expanded use of anti-bacterial agents may have resulted in alleviation of symptoms and signs. This situation may have thus reduced infectious disease cases that meet the FUO definition, while the development of imaging examinations may have improved the diagnosis rate of neoplasms.

When an affirmative diagnosis is difficult with the help of markers, etc., exclusion of other illnesses plays an important role for diagnosis. Fluorodeoxyglucose positron emission tomography has been increasingly reported to be useful for the diagnosis of FUO.[7] Although it is evident that the device is a powerful tool for the establishment of inflammatory pathological conditions, its applicability is currently limited to special facilities because of problems involving equipment investment and indication for children.

When an "FUO" case is presented, it is tempting to give priority to the establishment of a diagnosis by way of differentiating illnesses listed in the present survey. However, we consider it more important to evaluate the "severity" of the case on the basis of available information since the severity suggests the "morbid state" which in turn determines whether further appropriate examinations are required.

The present study has a limitation. Because this is a retrospective and multicenter study, there is a possibility of a recall bias about whether all data in all patients were included.

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Influence of polymorphisms within the methotrexate pathway genes on the toxicity and efficacy of methotrexate in patients with juvenile idiopathic arthritis

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5-aminoimidazole-4-carboxamide ribonucleotide transformylase, articular-type juvenile idiopathic arthritis, γ-glutamyl hydrolase, methotrexate

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Methotrexate (MTX), which causes adverse effects, such as liver and/or renal dysfunction, is the most common disease-modifying antirheumatic drug used for the treatment of rheumatoid arthritis and articular-type juvenile idiopathic arthritis (JIA).
- Pharmacogenetic studies analysing the MTX pathway genes would aid in the development of more personalized therapy.
- Results regarding the influence of gene polymorphisms on the toxicity and efficacy of MTX are conflicting, and there are marked differences between racial groups in pharmacogenetics.

WHAT THIS STUDY ADDS

- The non-TT genotype at γ-glutamyl hydrolase (*GGH*) T16C is associated with a high risk of liver dysfunction due to MTX, even after adjustment for duration of MTX treatment.
- Longer time interval from disease onset to MTX treatment and rheumatoid factor positivity are associated with lower efficacy of MTX in Japanese patients, as reported previously in Caucasian patients with JIA.

AIMS

We investigated whether several polymorphisms within the methotrexate (MTX) pathway genes were related to the toxicity and efficacy of MTX in 92 Japanese patients with articular-type juvenile idiopathic arthritis (JIA).

METHODS

Eight gene polymorphisms within the MTX pathway genes, namely, *RFC*, *BCRP*, *MTHFR* (two), *FPGS*, γ-glutamyl hydrolase (*GGH*; two) and *ATIC*, were genotyped using TaqMan assays. Liver dysfunction was defined as an increase in alanine transaminase to five times the normal upper limit. Non-responders to MTX were defined as patients refractory to MTX and were therefore treated with biologics.

RESULTS

The non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction ($P = 0.028$, odds ratio = 6.90, 95% confidence interval 1.38–34.5), even after adjustment for the duration of MTX treatment. A longer interval from disease onset to treatment (8.5 and 21.3 months, $P = 0.029$) and rheumatoid factor positivity ($P = 0.026$, odds ratio = 2.87, 95% confidence interval 1.11–7.39) were associated with lower efficacy of MTX.

CONCLUSIONS

The non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction, presumably because the C allele of *GGH* C16T may reduce the activity of GGH. The time interval before MTX treatment and rheumatoid factor positivity were associated with the efficacy of MTX treatment. The pharmacogenetics of the MTX pathway genes affects the toxicity and efficacy of MTX in Japanese JIA patients.

Introduction

Juvenile idiopathic arthritis (JIA) is one of the most common forms of paediatric chronic arthritis, with an incidence of approximately 9.7 per 100 000 children (aged 15 years and under) in Japan [1, 2]. Methotrexate (MTX) is the most common disease-modifying antirheumatic drug used for the treatment of articular-type JIA, namely the polyarticular- and oligoarticular-onset types of JIA [2]. Methotrexate is effective in about 75% of cases of the articular-type JIA, but causes adverse effects, such as liver and/or renal dysfunction [2, 3]. The effects of polymorphisms within the MTX pathway genes on the toxicity and efficacy of MTX in patients with rheumatoid arthritis (RA) and JIA have been studied [4–6].

The influence of polymorphisms within the MTX pathway genes encoding solute carrier family 19 member 1 (SLC19A1), also known as reduced folate carrier (RFC), 5,10-methylenetetrahydrofolate reductase (MTHFR), folypolyglutamate synthetase (FPGS), γ-glutamyl hydrolase (GGH), 5-aminomidazole-4-carboxamide ribonucleotide transformylase (ATIC) and breast cancer resistance protein (BCRP/ABCG2) on the toxicity and efficacy of MTX in patients with RA, JIA and other diseases has been studied [4–9]. However, results regarding the influence of these polymorphisms on the toxicity and efficacy of MTX are conflicting, and there are marked differences in pharmacogenetics between racial groups [10]. Therefore, we investigated whether polymorphisms within the MTX pathway genes were related to the toxicity and efficacy of MTX in 92 patients with articular-type JIA in Japan.

Patients and methods

Study population

Patients were eligible if they met the International League of Association for Rheumatology classification criteria for articular-type JIA [11]. A total of 92 children (74 girls and 18 boys; 12 with seronegative polyarticular onset, 46 with seropositive polyarticular onset and 34 with oligoarticular onset) in this study were treated at the Yokohama City University Hospital between December 2007 and December 2009.

All 92 patients had been treated with MTX for at least 3 months without biologics. Initially, MTX was administered orally at a dosage of 4–5 mg m^{-2} per week. Then the dosage was adjusted depending on tolerability and response (maximal dosage, 10 mg m^{-2} $week^{-1}$) [2]. Prednisolone was used concomitantly with MTX in 89 patients (96.7%). Folic acid supplementation was performed in nine patients (9.9%). Clinical data were collected from a patient's medical record without any knowledge of the individual's polymorphisms.

The study was performed in accordance with the Declaration of Helsinki, and approval for it was obtained from the Yokohama City University School of Medicine Ethics Committee. Each patient or his/her guardians gave written informed consent to participate in this study.

Definitions of toxicity and efficacy

For the evaluation of toxicity, liver dysfunction was defined as an increase in serum alanine transaminase (ALT) level to five times the normal upper limit before the addition of biologics.

Responders to MTX were defined as follows: (i) patients in whom the medication was terminated because they had remission of symptoms; (ii) patients who continued the treatment with stable doses of MTX; and (iii) patients who continued MTX treatment with the concomitant use of acceptable doses of prednisolone, without the addition of biologics, such as anti-tumour necrosis factor therapy [12] and anti-interleukin-6 receptor antibody therapy [13, 14].

Non-responders to MTX were defined as patients who were refractory to MTX and thus treated with biologics. Treatment with biologics was conducted according to the following criteria: (i) patients with a history of treatment with nonsteroidal anti-inflammatory drugs and MTX; and (ii) patients who had the active disease for at least 3 months after MTX treatment (up to 10 mg m^{-2} $week^{-1}$). Active disease was characterized by five or more swollen joints and three or more joints with limited range of movement accompanied by pain and/or tenderness, or the use of high doses of corticosteroids (>0.25 mg kg^{-1} daily), with accompanying unacceptable side-effects [12, 13].

Clinical predictors

Clinical predictors that may influence a patient's disease state and the toxicity and efficacy of MTX were selected on the basis of previous reports [5, 6, 15, 16]. The following factors were included: sex; age at disease onset; duration of MTX treatment; time interval from disease onset to MTX treatment; rheumatoid factor (RF) status; anti-cyclic citrullinated peptide (anti-CCP) status; and concomitant use of prednisolone and folic acid.

Genetic predictors

Genomic DNA was isolated from peripheral blood using the QIAamp DNA Mini kit (Qiagen K.K., Tokyo, Japan).

The following eight single nucleotide polymorphisms (SNPs) within the MTX pathway genes encoding RFC, MTHFR, FPGS, GGH, ATIC and BCRP were selected according to previous reports [4–9]. Genotyping for the SNPs of *RFC* G80A (rs1051266), *MTHFR* A1298C (rs1801131), *MTHFR* C677T (rs1801133), *FPGS* A1994G (rs10106), *GGH* C452T (rs11545078), *GGH* T16C (rs1800909), *ATIC* C347G (rs2372536) and *BCRP* C421A (rs2231142) was performed using the TaqMan assay (Applied Biosystems, Foster City, CA, USA). TaqMan SNP Genotyping Assays were used for *MTHFR* A1298C and *MTHFR* C677T, and Custom TaqMan SNP Genotyping Assays were used for *RFC* G80A, *FPGS*

A1994G, *GGH* C452T, *GGH* T16C, *ATIC* C347G and *BCRP* C421A [9] (see Supplementary data 1). These SNPs were analysed in real-time PCRs by the AB7500 Real Time PCR system (Applied Biosystems), in the conditions recommended by the manufacturer. Allele discrimination was performed using SDS software version 1.4 (Applied Biosystems).

Statistical analysis

For continuous predictors, such as age and duration of MTX treatment, Student's unpaired *t*-test was used to assess the association between clinical predictors and the toxicity and efficacy. For categorical predictors, such as genetic predictors and sex, a χ^2 test and Fisher's exact test were used to assess the association between predictors and the toxicity and efficacy. Possible confounding effects among the predictors were adjusted using a multiple logistic regression model.

Haplotype phases and haplotype frequencies were estimated using the Expectation-Maximization algorithm (minimum haplotype frequency >0.05). All statistical analyses were carried out using the SAS system version 9 (SAS Institute Inc., Cary, NC, USA).

Results

Distribution of the polymorphisms within the MTX pathway genes

The genotype frequencies for the eight SNPs under study were in Hardy–Weinberg equilibrium ($P > 0.05$). Each result was consistent with the findings of a previous report (see Supplementary data 2) [17].

The toxicity of MTX

Of 92 patients, 10 developed liver dysfunction. Methotrexate treatment of longer duration was a risk factor for liver dysfunction (104.3 months with liver dysfunction, 53.6 months without, $P = 0.005$). No other clinical variables were associated with liver dysfunction (Table 1). None of the patients with folic acid supplementation had liver dysfunction. However, this correlation of folic acid supplementation preventing liver dysfunction was not statistically significant, presumably because of the small study population.

Regarding the association between liver dysfunction and genetic predictors, the TT genotype at *GGH* T16C was a low risk factor for liver dysfunction [$P = 0.031$, odds ratio (OR) = 0.20, 95% confidence interval (CI) 0.03–0.98; Table 2 and Supplementary data 3]. In contrast, the non-TT genotype at *GGH* T16C was a high risk factor for liver dysfunction ($P = 0.031$, OR = 5.10, 95% CI 1.02–25.6), which is of significant clinical interest. This association was statistically significant even after adjustment for duration of MTX treatment ($P = 0.028$, OR = 6.90, 95% CI 1.38–34.5). None of the other SNPs was associated with liver dysfunction.

The *MTHFR* haplotypes and *GGH* haplotypes showed no significant association with liver dysfunction (data not shown).

The efficacy of MTX

Of 92 patients, 67 were non-responders to MTX. Delayed MTX treatment from disease onset (21.3 months with non-responders *vs.* 8.5 months with responders, $P = 0.029$) and RF positivity ($P = 0.026$, OR = 2.87, 95% CI 1.11–7.39) were risk factors for lower efficacy of MTX (Table 3). No other clinical variables were associated with efficacy.

Regarding the association between the efficacy of MTX and genetic predictors, there was no gene polymorphism significantly associated with efficacy (Table 4). The *MTHFR* haplotypes and *GGH* haplotypes showed no significant association with efficacy (data not shown).

In 64 patients treated with MTX within 1 year of disease onset, the CC genotype at *ATIC* C347G tended to be associated with lower efficacy. However, this was not statistically significantly after adjustment for the time interval and RF ($P = 0.106$, OR = 0.38, 95% CI 0.12–1.23) (Table 5).

Discussion

Several studies have shown the influence of polymorphisms within the MTX pathway genes on the toxicity and

Table 1

Association between clinical predictors and liver dysfunction

	ALT >5.0 times normal ($n = 10$)	ALT ≤5.0 times normal ($n = 82$)	*P* value
Age at onset (years, mean)	9.5	7.4	0.138
Sex (male)	20.0%	19.5%	0.971
Time interval from onset to treatment (months, mean)	17.7	17.9	0.987
Prednisolone	90.0%	97.6%	0.204
Folic acid	0.0%	11.0%	0.270
Duration of MTX treatment (months, mean)	104.3	52.6	0.005
MTX efficacy	30.0%	26.8%	0.832

ALT, alanine transaminase.

Table 2

Association between genetic predictors and liver dysfunction

Genotype	Allele model* OR†	*P* value	Dominant model* OR†	*P* value	Recessive model* OR†	*P* value
RFC G80A	1.51	0.414	0.21	0.121	0.59	0.627
BCRP C421A	1.05	0.930	0.80	0.840	0.99	0.988
MTHFR C677T	1.45	0.451	1.12	0.896	2.28	0.214
MTHFR A1298C	0.89	0.852	1.08	0.539	0.74	0.655
FPGS A1994G	0.54	0.249	4.88	0.068	0.70	0.600
GGH T16C	0.42	0.118	0.83	0.475	0.20	0.031
GGH C452T	0.61	0.506	–	–	0.61	0.502
ATIC C347G	1.40	0.560	0.48	0.814	1.17	0.336

M, major allele; and m, minor allele. Major alleles are the A allele at *RFC* G80A, C allele at *BRCP* C421A, C allele at *MTHFR* C677T, A allele at *MTHFR* A1298C, G allele at *FPGS* A1994G, T allele at *GGH* T16C, C allele at *GGH* C452T and C allele at *ATIC* C347G. *Allele model: M *vs.* m; dominant model, (MM or Mm) *vs.* mm; recessive model, MM *vs.* (Mm or mm). †Non-adjusted odds ratio.

Table 3

Association between clinical predictors and methotrexate efficacy

	Responder ($n = 25$)	Non-responder ($n = 67$)	*P* value
Age at onset (years, mean)	6.6	7.9	0.180
Sex (male)	12.0%	22.4%	0.264
Time interval from onset to treatment (months, mean)	8.5	21.3	0.029
Prednisolone	96.0%	97.0%	0.807
Folic acid	4.0%	11.9%	0.254
C-reactive protein at start of treatment (mg dl^{-1}, mean)	2.8	3.3	0.685
Anti-cyclic citrullinated peptide [>4.5 (U ml^{-1})]	32.0%	55.2%	0.062
Rheumatoid factor [>14 (IU ml^{-1})]	40.0%	65.7%	0.026

Table 4

Association between genetic predictors and methotrexate efficacy

Genotype	Allele model* OR†	*P* value	Dominant model* OR†	*P* value	Recessive model* OR†	*P* value
RFC G80A	1.01	0.979	1.32	0.572	1.61	0.435
BCRP C421A	1.28	0.496	0.24	0.151	0.99	0.979
MTHFR C677T	0.75	0.399	0.79	0.708	0.42	0.115
MTHFR A1298C	1.05	0.918	0.36	0.282	0.87	0.775
FPGS A1994G	0.95	0.900	1.37	0.726	1.01	0.984
GGH T16C	1.01	0.986	2.83	0.294	1.24	0.654
GGH C452T	1.15	0.805	–	–	1.15	0.805
ATIC C347G	0.65	0.237	1.08	0.931	0.50	0.139

*Allele model: M *vs.* m.; dominant model, (MM or Mm) *vs.* mm; recessive model, MM *vs.* (Mm or mm). †Non-adjusted odds ratio.

efficacy of MTX in patients with RA [4, 8, 9]. However, results are conflicting, and there are marked differences between racial groups in pharmacogenetic studies [10]. We could find only two studies on the pharmacogenetics of MTX in patients with JIA in Caucasian patients [5, 6], but not one in an Asian population. This is the first reported study on pharmacogenetics of MTX in patients with JIA in an Asian population.

First, we found that the non-TT genotype at *GGH* T16C was associated with a high risk of liver dysfunction. This should be taken into consideration in treating patients carrying the non-TT genotype at *GGH* T16C with MTX in order to prevent liver dysfunction.

Once inside the cell, MTX undergoes FPGS-catalysed polyglutamation by the addition of two to seven glutamic acid groups. The polyglutamated form is not

Table 5

Association between *ATIC* 347CC genotype and methotrexate efficacy in patients with the early phase of juvenile idiopathic arthritis

(a)	OR†	95% Confidence interval	*P* value
ATIC 347CC genotype	0.32	0.11–0.93	0.033

(b)	OR‡	95% Confidence interval	*P* value
ATIC 347CC genotype	0.38	0.12–1.23	0.106
Rheumatoid factor [>14 (IU ml^{-1})]	0.22	0.07–0.72	0.012
Time interval*	0.85	0.70–1.04	0.12

*Time interval, time interval from disease onset to methotrexate treatment. †Non-adjusted odds ratio. ‡Adjusted odds ratio.

readily transported across the cell membrane, and thus, the intracellular half-life of MTX is increased. This polyglutamation process is reversed via GGH-catalysed removal of the glutamic acid groups. Therefore, the amount of intracellular MTX-polyglutamates (MTX-PGs) depends on the net rate of polyglutamation determined by the opposing activities of FPGS and GGH [8].

It was reported that *GGH* T16C, which results in a Cys6Arg substitution, was associated with the efficacy of MTX in patients with RA. The variant C allele may cause a loss of GGH activity, resulting in decreased efflux of MTX and thus increased intracellular MTX-PG levels [8]. This result was consistent with ours. Although we did not address the MTX-PG levels in hepatic cells, it is possible that the C allele at *GGH* T16C was associated with reduced GGH activity and thereby increased the MTX-PG levels in hepatic cells. As a result, the risk of liver dysfunction rises. The AA genotype at *FPGS* A1994G tended to be associated with liver dysfunction ($P = 0.068$, OR = 4.88, 95% CI 0.78–30.9). Future research using large study populations to address the effects of the combination of *GGH* and *FPGS* polymorphisms on MTX toxicity is needed.

The MTX dosage was probably associated with the toxicity and efficacy of the drug. In this cohort study, some patients underwent MTX treatment at other hospitals and had liver dysfunction before being referred to our institution. For these patients, we did not have access to previous medical records concerning the exact dosage of MTX at the time of liver dysfunction. As a general rule, non-responders to MTX received higher dosages of MTX (up to 10 mg m^{-2}) before the introduction of biologics than the responders. We therefore used MTX efficacy as the clinical predictor instead of MTX dosage. The MTX efficacy tended to be associated with liver dysfunction ($P = 0.083$), although the effect of MTX dosage on the toxicity and efficacy of this drug should be evaluated directly in the future.

Second, we found that the longer time interval from disease onset to MTX treatment and RF positivity were associated with lower efficacy of MTX. This was consistent with previous research results. Time to treatment was reported as an important factor in the response to MTX in patients with JIA [6], and RF positivity was associated with worse disease activity [18, 19].

Paediatric rheumatologists have recently been able to use MTX for patients with earlier phases of JIA, because MTX has become well known as a first-line drug in the treatment of RA and JIA [2, 3]. Therefore, we analysed the subgroup of early JIA patients. In those who were treated with MTX within 1 year of disease onset, the CC genotype at *ATIC* C347G tended to be associated with the lower efficacy of MTX. Methotrexate-polyglutamates inhibit ATIC, the last enzyme in the *de novo* purine synthesis pathway. Methotrexate achieves part of its anti-inflammatory effect through inhibition of ATIC, which results in the release of the anti-inflammatory agent, adenosine [9].

It was reported that RA patients with the G allele at *ATIC* C347G, resulting in a Thr116Ser substitution, were likely to have a good response to MTX [9]. Although the effect of the C347G polymorphism on ATIC enzyme activity is unknown, *ATIC* C347G may be in linkage disequilibrium with an unknown functional variant, which is associated with the activity of the purine synthesis pathway and with the level of adenosine production. Future basic and clinical prospective studies on a large number of patients are needed to elucidate this association.

There are some limitations to the present study. The incidence of RF positivity in the patient population studied was higher than generally seen (~10%) [18], presumably because our institution is one of the very few paediatric rheumatology centres in Japan, and many severe cases with RF positive are referred to our institution for highly specialized treatment with biologics [13, 20]. The efficacy rate of MTX in this study (28%) was significantly lower than those in previous Japanese reports [2, 3]. This may be due to the use of a new second-line choice of biologics, as well as the characteristics of our institution and the lower limit of the maximal MTX dosage (10 mg m^{-2}) for the treatment of JIA in Japan [2].

In summary, we found an association between the non-TT genotype at *GGH* T16C and liver dysfunction due to MTX. We also found an influence of time interval from disease onset to MTX treatment on the efficacy of MTX in Japanese patients with JIA. Our study showed the importance of early use of MTX for patients with JIA as well as the possibility of more personalized therapy for patients with JIA based on pharmacogenetic study of the MTX pathway genes.

Competing Interests

There are no competing interests to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary data 1
TaqMan® SNP Genotyping Assays.
Supplementary data 2
Distribustion of gene polymorpshisms under the study.
Supplementary data 3
Distribustion of gene polymorpshisms in patiets with or without liver dysfunction.

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EXPERT REVIEWS

Tocilizumab: molecular intervention therapy in children with systemic juvenile idiopathic arthritis

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Systemic juvenile idiopathic arthritis (JIA) is a subtype of chronic childhood arthritis of unknown etiology, manifested by long-lasting systemic inflammation and complicated by joint destruction, functional disability and growth impairment. Macrophage activation syndrome is the most devastating complication, which is associated with serious morbidity. IL-6 has been hypothesized to be a pathogenic factor of this disease. The anti-IL-6 receptor monoclonal antibody, tocilizumab, was developed, and we investigated the safety and efficacy of tocilizumab in children with this disorder. The Phase II trial revealed that high-grade fever abruptly subsided and that inflammatory markers were also normalized. The dose of tocilizumab for systemic JIA was revealed to be 8 mg/kg at 2-week intervals. The Phase III trial, a placebo-controlled, double-blind study, indicated that patients in the tocilizumab group had sustained clinical measures of effectiveness and wellbeing, whereas most of those in the placebo group needed rescue treatment. The most common adverse events were symptoms of mild infections and transient increases of alanine aminotransferase. Serious adverse events were anaphylactoid reaction and gastrointestinal hemorrhage. Clinical and laboratory improvement in fever, sickness behavior, C-reactive protein gene expression and chronic inflammatory anemia in children with systemic JIA treated with tocilizumab indicated the possible roles played by IL-6 in this inflammatory disease. Thus, tocilizumab is generally safe and well tolerated. It might be a suitable treatment in the control of this disorder, which has so far been difficult to manage.

KEYWORDS: biologic response modifier • C-reactive protein • IL-6 • IL-6 receptor • systemic juvenile idiopathic arthritis • tocilizumab

Systemic juvenile idiopathic arthritis (JIA), a systemic inflammatory disease of unknown etiology, is one of the most common physically disabling conditions of childhood [1]. The long-lasting inflammation also causes anemia, impairment of growth and development, and amyloidosis. Moreover, the acute complication known as macrophage activation syndrome (MAS) is associated with serious morbidity and sometimes death [2].

This severe inflammatory disease is refractory to various cytotoxic and immunosuppressive medications. High doses and a long duration of corticosteroids have been inevitably chosen as regimens for suppressing disease activity. Consequently, corticosteroid therapy leads to iatrogenic Cushing-like syndrome, osteoporosis and compression fractures, growth impairment, cataracts and increased susceptibility to overwhelming infection [3].

The pathogenesis of systemic JIA remains obscure. However, several studies have provided evidence implicating the circulating levels of IL-6 and soluble IL-6 receptor (sIL-6R), but not TNF-α, as playing an essential role as inflammatory mediators. The oldest cytokine, IL-1β, has also been recognized as an important pathogenic player in systemic JIA [4]. The impaired natural killer (NK) cell function correlated with perforin gene (*PRF1*) mutation [5] and defective phosphorylation of IL-18 receptor-β [6] was also reported.

Serum IL-6 and IL-6R levels in children with systemic JIA have shown correlations with both disease activity and the extent and severity of

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joint involvement [7]. A human *IL-6* gene transgenic study in mice indicated that overproduction of IL-6 leads to severe inflammatory responses and growth retardation, similar to that found in children with systemic JIA [8]. Taken together, this information indicates that overfunction of the IL-6 signaling system may play a central role in the induction and progression of systemic JIA and its complications. This disease is starting to be regarded as an autoinflammatory disease rather than an autoimmune disease [9].

Recently, the molecular mechanisms of inflammatory responses have been precisely described, and proinflammatory cytokines are known to contribute to variable physiologic and pathophysiologic processes of inflammation. Among their physiologic functions, cytokines may regulate the central mechanisms of fever and sickness behavior such as prolonged sleep, lethargy and anorexia observed in experimental animals [10]. The combination of IL-1β and IL-6 plays an essential role in the anemia of chronic inflammatory diseases [11]. IL-6 can function as the key hepatocyte-stimulating factor to induce, at least in rodents, acute-phase reactants including fibrinogen, α-2-macroglobulin and α-1-acid glycoprotein [12]. Serum amyloid A (SAA) [13] and C-reactive protein (CRP) [14] are also products of the IL-6 plus IL-1β action in human cell line experiments. Overproduction of IL-6 has been implicated in the disease pathology of several inflammatory autoimmune disorders, rheumatoid arthritis [15], Castleman's disease [16] and adult Still's disease [17]. However, direct evidence in humans is not yet available to show that the inflammatory changes of clinical manifestations and laboratory findings are correlated with cytokine functions.

Tocilizumab (Actemra®, Roche, Basel, Switzerland) is a recombinant humanized anti-IL-6R monoclonal antibody that acts as an IL-6 antagonist [18]. The hypothesis that inhibition of IL-6 signaling with tocilizumab can result in a significant improvement in the signs and symptoms of systemic JIA appears to have been substantiated in Phase II [19] and Phase III [20] clinical trials for children with systemic JIA, which demonstrated a marked reduction in inflammatory responses and an improvement in osteoporosis and growth retardation. The results of these clinical trials indicate that tocilizumab treatment generally has a good safety profile and improves health-related quality of life in children with systemic JIA. Tocilizumab appears to provide an additional option for those children who have recurrent inflammatory episodes. In addition, the blockade of IL-6R by the monoclonal antibody tocilizumab has a distinct mechanistic action on the IL-6 signaling pathway, that is, molecular intervention. Thus, the alterations in clinical manifestations and laboratory findings during tocilizumab treatment can be attributable to the normalization of IL-6 and sIL-6R levels, indicating that clinical inflammatory manifestations such as fever, sickness behavior, osteoporosis and growth retardation, and laboratory abnormalities such as increased levels of acute-phase proteins and chronic anemia are direct or indirect functions of the IL-6 signaling pathway.

Overview of current therapy

Children with systemic JIA have a higher rate of etanercept failure than other chronic arthritis subtypes, indicating that TNF-α is not the only cytokine implicated in the pathogenesis of the disease [21]. Although serum concentrations of IL-1 are not increased in this disease, dysregulation of IL-1 might play a part in the pathogenesis [22]. Case reports and an early uncontrolled study have suggested that treatment with anakinra, an IL-1 receptor antagonist, might be effective in patients with this illness, but MAS still occurred despite treatment with anakinra [23]. Recently, a trial of anakinra for patients with systemic JIA was carried out in France, and less than half of the patients achieved a marked and sustained improvement [24]. Anakinra has not been approved for patients with systemic JIA by the government in either Japan or the USA. Thus, tocilizumab is the only approved drug for children with systemic JIA in Japan. Fortunately, trials of tocilizumab for patients with systemic JIA are now making progress in the EU and the USA, and thus, in the near future, tocilizumab will hopefully be approved and available worldwide.

Clinical & laboratory features of systemic JIA

Systemic JIA

Children with JIA represent a clinical heterogeneity of phenotypes. According to the ILAR classification criteria (Edmonton, 2001) [25], the systemic type of JIA is one of the JIA subtypes, which is unique among the chronic arthritides of childhood in several ways. In particular, the range and severity of characteristic extra-articular features mark this disease as a systemic inflammation with arthritis [1].

Systemic inflammatory manifestations with recurrent quotidian fever, fatigue, anorexia, skin rash and polyarthritis are present and are sometimes accompanied by serositis, lymphadenopathy and hepatosplenomegaly. Laboratory investigation shows markedly increased levels of CRP, SAA and other acute-phase reactants [1].

In the long-term course of the disease, severe arthritis progresses in half of the affected children, is resistant to treatment and can eventually result in significant disability [26]. Moreover, growth retardation, severe osteoporosis and compression are seen in most patients, and x-ray examinations and laboratory experiments suggest that enchondral ossification may be disturbed by the long-lasting inflammation. *In vitro* examination of IL-6 on ATDC5 cells, which are chondrogenic progenitor cells, indicated that IL-6 inhibits the early chondrogenesis of these cells. It was suggested that IL-6 might affect committed stem cells at a cellular level during chondrogenic differentiation of growth plate chondrocytes [27]. In these children, laboratory examination will frequently indicate anemia, hypoalbuminemia and hypergammaglobulinemia of chronic inflammatory disease. Consequently, children with recurrent inflammatory episodes develop amyloidosis [28]. Thus, the emerging consensus in the field of pediatric rheumatology is that since the clinical abnormalities and pathogenesis of systemic JIA are attributable to a breakdown of proinflammatory cytokine homeostasis, this disease should be viewed as an autoinflammatory syndrome rather than an autoimmune disease [9].

Macrophage activation syndrome

The most devastating complication of systemic JIA is MAS [2]. Approximately 7% of affected children progress to MAS, which is associated with serious morbidity and sometimes death. It can

be considered to be a process of the disease rather than a disease itself due to hypercytokinemia [29]. It can be difficult to diagnose patients as having MAS at a given time because MAS is a disease in which a series of events such as thrombocytopenia, endothelial cell damage, coagulation abnormalities, mitochondrial permeability transition and multiple organ failure occurs, fades away, and worsens in a couple of days.

Macrophage activation syndrome is clinically characterized by the rapid development of fever, hepatosplenomegaly, lymphadenopathy, purpura and mucosal bleeding. In our experience, an exact diagnosis will be made when precise laboratory examinations are performed during the course of the process. Laboratory studies primarily indicate the presence of hematocytopenia, and then, combinations of serum β2-microglobulin and ferritin, elevated tissue-derived enzymes such as mitochondrial aspartate aminotransferase (mAST), lactate dehydrogenase (LDH) and creatine phosphokinase (CK), hypoalbuminemia, increased levels of fibrin degradation products (FDP-E, D-dimer) and elevated triglycerides. A bone marrow examination, if performed with proper timing, may show active phagocytosis by macrophages and histiocytes [30]. Accompanying the progression of the process, finally, increases in creatinine, alanine aminotransferase (ALT) and amylase levels are present, indicating multiple organ failure.

The pathogenesis of MAS remains to be established. The first report described the pathogenic role of TNF-α in MAS [2]. The increased levels of IFN-α, TNF-α and other proinflammatory cytokines correlate with the rapid development of clinical symptoms and the progression of abnormal laboratory parameters [31]. In addition, since systemic JIA patients display decreased levels of perforin in NK cells and diminished NK cell function, the recent investigation suggested that perforin gene (*PRF1*) mutations also play a role in the development of MAS in systemic arthritis patients [5]. Thus, MAS would be the transition form of the disease process from IL-6 cytokinemia in systemic JIA to multiple proinflammatory cytokinemia for the background of *PRF1* gene mutation and diminished NK cell function.

Biologic function of IL-6 & tocilizumab

IL-6 is one of the most pleiotropic cytokines known that is involved in regulating a wide variety of inflammatory and immune functions, B-cell differentiation, T-cell growth, acute-phase reactions and hematopoiesis [32,33].

The first step in the induction of the transduction signals by IL-6 is the binding to its IL-6R, which is either localized at the cell surface or present in a soluble form in serum. The association of the IL-6/IL-6R complex with another receptor, gp130, forms a high-affinity complex that triggers specific transduction signals. Three members of the janus kinase family, JAK1, JAK2 and TYK2, are closely related to gp130 and are rapidly activated in the presence of IL-6 [34]. These kinases phosphorylate the tyrosine residues of the gp130 cytoplasmic domain, which allows the recruitment and phosphorylation of transcriptional factors of the signal transducers and activators of transcription family (STAT1 and STAT3) [35]. Once activated, the STAT proteins may activate different genes. Thus, the blockade of IL-6R by tocilizumab can result in invalidity of the formation of phosphorylated STAT proteins, which inhibits inflammatory responses [36].

Pathogenesis of systemic JIA & MAS

The pathogenic role of proinflammatory cytokines in systemic JIA has long been investigated. IL-6 is reported to be markedly elevated in blood and synovial fluid [37]. The IL-6 level increases before each fever spike and correlates with the systemic activity of the disease, arthritis and an increase in acute-phase reactions [38]. Abnormalities in the regulation of IL-6 are also responsible for the thrombocytosis and anemia seen in this disease [7]. *In vitro* studies have documented increased production of IL-6 by peripheral blood mononuclear cells from patients with systemic JIA [39]. An imbalance in IL-6 homeostasis is suggested by the observations that sIL-6R concentrations are significantly increased in children with systemic JIA. Growth retardation was found in IL-6 transgenic mice overexpressing human IL-6, similar to that in children with systemic JIA [8]. In contrast to IL-6, TNF-α levels are not increased in systemic JIA. Taken together, IL-6 and IL-6R might play a central role in the induction and progression of systemic disease and its complications. However, direct evidence in humans is not yet available.

During the course of recurrent inflammatory episodes of systemic JIA, MAS often follows a viral infection, such as Epstein–Barr virus or influenza virus [40]. Changes in medications, as well as the introduction of nonsteroidal anti-inflammatory drugs, gold compounds or methotrexate, were reported to trigger the syndrome [41]. However, it seems likely that changes in medications were coincidental, that is, occurring in a child who was susceptible to MAS and who required additional therapy for uncontrolled systemic JIA. The histopathologic features of skin biopsy specimens are the presence of microthrombi and endothelial cell proliferation [42], indicating that due to overwhelming proliferation of various proinflammatory cytokines such as IFN-γ and TNF-α, continuing damage to endothelial cells and the resultant vasculitis induce disseminated intravascular coagulation (DIC) and, subsequently, multiple organ failure. This is the whole spectrum of clinical MAS.

Clinically, MAS starts with thrombocytopenia and leukocytopenia, and then abrupt improvements in erythrocyte sedimentation rate (ESR) and CRP levels can be seen. Fibrin degradation products (FDP-E, D-dimer) and hypofibrinogenemia are present, indicating DIC due to activated and destroyed endothelial lining of the vasculature by combinatorial effects of proinflammatory cytokines [43]. Markedly increased levels of cytokine-induced proteins, serum ferritin by TNF-α [44] and β2-microglobulin by IFN-γ [45] can be observed during this stage. Subsequently, rising levels of serum mAST, LDH and CK indicate apoptosis due to mitochondrial permeability transition by TNF-α [46], which can solely be protected by cyclosporine [47]. In the late phase of MAS, increased levels of triglycerides and decreased levels of total cholesterol are present due to inhibited lipoprotein lipase activity by TNF-α [48]. Finally, multiple organ failure along with DIC will progress.

Clinical signs & symptoms seen in patients with systemic JIA & proinflammatory cytokines

Fever & sickness behavior in systemic inflammation

During the clinical trials, the most prominent features of the effects of tocilizumab were the abrupt normalization of fever and disappearance of fatigue, lethargy or anorexia in children with long-lasting inflammation with regard to systemic JIA [19,20].

Systemic inflammation is accompanied by changes in body temperature and behavior. The proinflammatory cytokines, IL-1β, IL-6 and TNF-α, synthesized by activated macrophages in response to experimental administration of the bacterial pyrogen lipopolysaccharide (LPS), are considered important mediators of fever and sickness behavior [6].

Recent investigations revealed that experimental fevers are generally polyphasic, and that different mechanisms underlie different febrile phases [49]. Signaling mechanisms of the most common pyrogen used, LPS, have been found to involve Toll-like receptor 4 [50]. The roles of endogeneous cytokines, particularly IL-6 and the cytokine-like hormone leptin, but not IL-1β or TNF-α, have been confirmed by using cytokine-specific antisera to be the key mediators of fever and sickness behavior assessed by voluntary exercise and food intake induced by LPS in rats [51].

Proinflammatory cytokines are then switched to a downstream mediator, prostaglandin (PG)E2 [52]. An indispensable role of PGE2 in the febrile response to LPS has been demonstrated in studies with targeted disruption of genes encoding either PGE2-synthesizing enzymes or PGE2 receptors. EP3 (a G-protein-coupled receptor) is likely to be the primary fever receptor, and the effector pathways of fever start from EP3-bearing neurons [38]. The neurons project to the raphe pallidus in the hypothalamus. Inflammatory signaling and thermoeffector pathways involved in fever and sickness behavior are further modulated by neuropeptides and peptide hormones such as leptin [51].

Tocilizumab is an IL-6R-specific monoclonal antibody, and clinical studies involving children with systemic JIA have demonstrated the targeted blockage of the IL-6 signaling pathway in humans. Consequently, tocilizumab treatment attenuates the body temperature rise and sickness behavior, indicating that the major manifestations of this systemic inflammatory disease are apparently IL-6 related. Skin rash, which appears to accompany a rise in body temperature, could be supposed to be an IL-6-related skin manifestation of systemic JIA, but this topic needs further investigation.

C-reactive protein

C-reactive protein is an acute-phase protein and a sensitive marker and mediator of inflammation in the clinical setting. CRP was used as one of the surrogate markers for assessing the flares of the disease in tocilizumab trials. The administration of tocilizumab to children with systemic JIA rapidly attenuated the increased levels of CRP in serum within a few days.

The synergistic induction mechanism of *CRP* gene expression by IL-1β and IL-6 in the human hematoma cell line, Hep3B cells, was recently investigated [14,53]. In the early induction phase, IL-1β and IL-6 activate NF-κB p65 and the janus kinase family, respectively. The activation of janus kinases by IL-6 allows the recruitment and phosphorylation of the transcriptional factor, STAT3. NF-κB p65 forms a complex with STAT3, which inhibits expression of the *CRP* gene. In the late induction phase, synergistic stimulation by IL-1β and IL-6 causes the formation of a heterodimeric complex with c-Fos, STAT3 and hepatocyte nuclear factor (HNF)-1-α, which in turn induces synergistic expression of the *CRP* gene. Thus, transcriptional complex formation of c-Fos/STAT3/HNF-1α plays an essential role in cytokine-driven *CRP* gene expression. Tocilizumab treatment, therefore, ameliorates the CRP rise by inhibiting the formation of the transcriptional complex.

Chronic anemia

Anemia occurs in patients with chronic inflammatory disorders such as infection, autoimmune disease or chronic kidney disease [11]. Anemia of chronic disease is characterized by normocytic or microcytic iron-deficiency anemia and preserved marrow iron. Proinflammatory cytokines, particularly IL-6, are believed to have an important role in this syndrome.

Early clues regarding the role of IL-6 in the pathogenesis of chronic inflammatory anemia were discovered in the cancer setting [54], where IL-6 has been evaluated as an antitumor immunotherapy. In patients with advanced ovarian cancer, anemia was so severe that the IL-6 level was the only factor other than disease stage to independently predict hemoglobin levels in a multivariate analysis. In addition, administration of human recombinant IL-6 was found to induce a rapid-onset, dose-dependent, progressive form of anemia that was quickly reversible after cessation of therapy [55].

Recent studies revealed the role of IL-6 in chronic anemia [56]. During acute-phase reactions, proinflammatory cytokines impair iron metabolism, particularly plasma iron turnover and ferritin synthesis, with the result that patients with acute or chronic infections have lower serum iron, lower tranferrin saturation and higher ferritin concentrations than do persons without apparent inflammation.

As a key regulator of transmembrane iron transport, hepcidin controls the absorption of iron in the intestine, the mobilization of iron from hepatic stores and iron recycling by macrophages [56]. At the first stage, inflammation leads to macrophage activation to produce IL-6, which acts on hepatocytes to induce hepcidin production. Under the influence of elevated hepcidin concentrations, hepcidin inhibits macrophage iron release and intestinal iron absorption, leading to hypoferremia, which limits the availability of iron for erythropoiesis, thereby contributing to the anemia associated with inflammation.

During inflammation, IL-6, but not IL-1β or TNF-α, rapidly induces hepcidin synthesis in human hepatocytes and corresponding hypoferremia. Anti-IL-6 antibodies block the induction of hepcidin mRNA in primary human hepatocytes treated with the bacterial endotoxin, LPS [57]. IL-6-knockout mice failed to produce hepcidin in response to inflammatory challenges [58]. In our studies, chronic anemia was gradually improved in association with the blockade of the IL-6 signaling pathway by tocilizumab. Thus, IL-6 is presumably the most potent cytokine of chronic inflammatory anemia in children with systemic JIA.

Development of tocilizumab as a blocking agent of the IL-6 signaling pathway

Tocilizumab is a genetically engineered monoclonal antibody of the IgG1 subclass that was humanized by the technique of complementary-determining region grafting from mouse anti-human IL-6R monoclonal antibody [18,59]. Tocilizumab binds to both membrane-bound and sIL-6R and inhibits the formation of the IL-6/IL-6R complex that results in a decrease in signal transduction via gp130. The IL-6R molecule is theoretically the exclusive target of tocilizumab, and thus, the roles of the IL-6 signaling pathway in inflammatory immune diseases can be clearly elucidated when tocilizumab is administered in humans as a therapeutic agent. Thus, tocilizumab treatment can be termed a 'molecular intervention.'

The use of intravenous tocilizumab in patients with rheumatoid arthritis was investigated and found to be more effective than placebo in reducing disease activity and to have a safety profile consistent with that of other biological and immunosuppressive therapies [60]. Notably, tocilizumab appears to provide an additional option for those patients who do not respond sufficiently to methotrexate and other biological response modifiers. In turn, tocilizumab administration proved that IL-6 is the key pathogenic cytokine in the induction and progression of rheumatoid arthritis. Together with evidence that targeting and inhibiting TNF-α with infliximab or etanercept can result in significant improvement in signs and symptoms of rheumatoid arthritis, the combined role of IL-6, TNF-α and probably IL-1β rather than the single independent action of each cytokine in inflammatory responses might be important. In other words, a vicious cycle is formed by the combination of these proinflammatory cytokines from a pathologic viewpoint, causing progression and continuation of inflammatory responses in rheumatoid arthritis. Thus, in many patients but not all, any monoclonal antibodies or specific receptors can block the overwhelming joint inflammation.

Efficacy of tocilizumab for children with systemic JIA

Phase II trial

The Phase II clinical study of tocilizumab was conducted in children with severe and active systemic JIA refractory to high-dose, long-term corticosteroids to investigate the safety, tolerability, antigenicity, pharmacokinetics and efficacy of the drug [19]. Eight boys and three girls were enrolled. At enrollment, these 11 children were between 3 and 18 years of age. The median duration of the systemic disease was 3.8 years. The mean number of active joints was 4.5. Six of them exhibited severe growth retardation and osteoporosis with complicating compression vertebrae fractures. CRP and ESR values were high and the white blood cell count was over 15,000/ml. The profile of proinflammatory cytokines and soluble receptors in serum was investigated, and IL-6 and sIL-6R, but not IL-1β and TNF-α, were persistently detected at high levels, indicating the failure of IL-6 signaling pathway homeostasis.

The study, designed as a dose-escalating trial, began with three infusions of tocilizumab 2 mg/kg at 2-week intervals. When the CRP value was demonstrated to be positive at least 5 days after the initial and second administrations of tocilizumab, the dose was increased to 4 mg/kg and was administered three times every 2 weeks. If CRP levels did not improve, then three infusions of 8 mg/kg were administered at 2-week intervals. Assessment of disease response was made according to American College of Rheumatology (ACR) Pedi responses to the following six items [61], although it is not yet validated for systemic JIA: physician's and patients'/parents' general assessment on a 10 cm visual scale, functional ability, number of active joints, number of joints with restriction of motion, and CRP/ESR values (the original ACR Pedi used the ESR as a laboratory parameter instead of CRP).

After the first administration of tocilizumab, high-grade or quotidian fever abruptly subsided and vague complaints such as fatigue, lethargy and anorexia disappeared. Severe arthritis improved in all 11 children within a few weeks. Laboratory examinations revealed that CRP and ESR levels had returned to the normal range. Ten out of the 11 children improved at 2 weeks after the first administration of tocilizumab as assessed by ACR Pedi 30/50% responses. Before the second administration, eight children had increases in CRP and 4 mg/kg of tocilizumab was infused. Three of these eight children had subsequent elevations in CRP and, consequently, three infusions of 8 mg/kg tocilizumab were administered, with no further increases in the CRP value. Overall improvement in arthritis and systemic features assessed by an ACR Pedi 30, 50 and 70% improvement was 90.9, 90.9 and 63.6%, respectively. In general, tocilizumab was well tolerated. No patient withdrew during the study period. The adverse events were upper respiratory tract infection, pustules on extremities and eczema. All laboratory abnormalities were mild and no serious events requiring urgent treatment were noted.

Results of the Phase II trial suggested that although 2–4 mg/kg of tocilizumab could suppress disease activity, 8 mg/kg is probably required to control disease activity in children with systemic JIA. Clinical manifestations such as quotidian fever, fatigue, lethargy and anorexia, and laboratory abnormalities such as increased levels of CRP and ESR seen in active disease disappeared with tocilizumab treatment by blocking IL-6R alone, indicating that the IL-6 signaling pathway is directly implicated in the pathogenesis of this disease.

Phase III trial

The Phase III trial was conducted to investigate the safety and efficacy of tocilizumab for children with systemic JIA who were refractory to conventional treatment [20]. The study consisted of three phases: an open-label lead-in phase of 6 weeks, a double-blind, randomized, placebo-controlled phase of 12 weeks, and an open-label extension phase of at least 48 weeks. Tocilizumab was administered intravenously at 8 mg/kg, every 2 weeks. The primary end points in the open-label phase were the proportion of children achieving an ACR Pedi 30% improvement and the proportion of those with a reduction of CRP concentration to less than 5 mg/l. Children who achieved ACR Pedi 30% responses and low CRP concentrations were randomly assigned

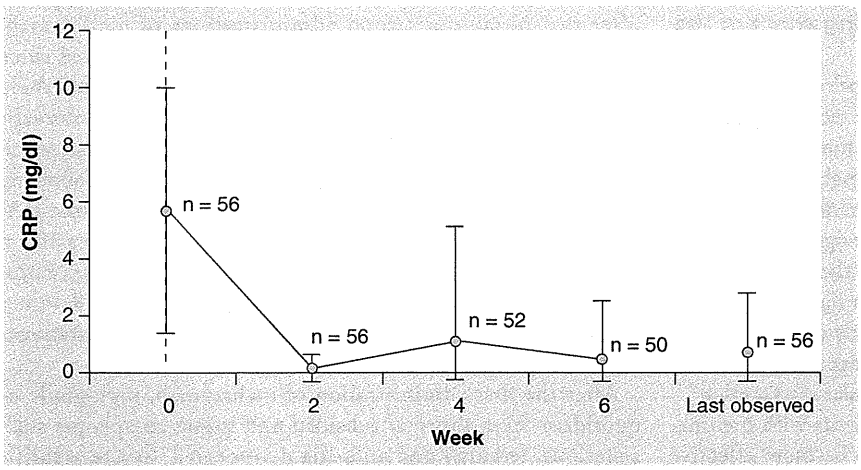

Figure 1. Effects of tocilizumab on C-reactive protein value. Increased level of CRP was decreased rapidly to the normal range after the first administration of tocilizumab. Vertical lines represent the standard error bars.
CRP: C-reactive protein.
Data from Phase III trial of tocilizumab for patients with systemic juvenile idiopathic arthritis [20].

to receive an infusion of tocilizumab 8 mg/kg or placebo every 2 weeks in a double-blind manner. Children who did not maintain an ACR Pedi 30% response or those whose CRP concentrations increased to at least 15 mg/l were withdrawn for rescue medication.

Initially, 56 children with severe systemic JIA were enrolled in the open-label study and six patients were withdrawn; three had anti-tocilizumab IgE antibody, two had adverse events (anaphylactoid reaction and gastrointestinal hemorrhage) and one was withdrawn because of absence of efficacy. Six patients did not meet the response criteria for randomization for the double-blind phase. In the double-blind phase, one patient was excluded because of being disqualified. The study mask for this patient was broken by mistake and pharmacokinetic data were unexpectedly unmasked. Therefore, 43 patients were included in the efficacy analysis. Overall, 23 children were placed in the placebo group and 20 children in the tocilizumab group in the 12-week double-blind phase. One patient was withdrawn from each treatment group in the double-blind phase because of adverse events (leaving a total of 41 patients). In the extension phase, nine patients previously withdrawn from the open-label and the double-blind phases were re-enrolled and a total of 48 children completed the 48-week open-label extension study.

Again, high-grade or quotidian fever abruptly subsided, and vague complaints disappeared after the administration of tocilizumab (Figure 1). At the end of the open-label phase, ACR Pedi 30, 50 and 70% responses were achieved by 91, 86 and 68% of the enrolled children, respectively. In the double-blind, placebo-controlled phase, 17% of children in the placebo group maintained an ACR Pedi 30% response and CRP concentrations of less than 15 mg/l compared with 80% of children in the tocilizumab group, indicating the remarkable efficacy of

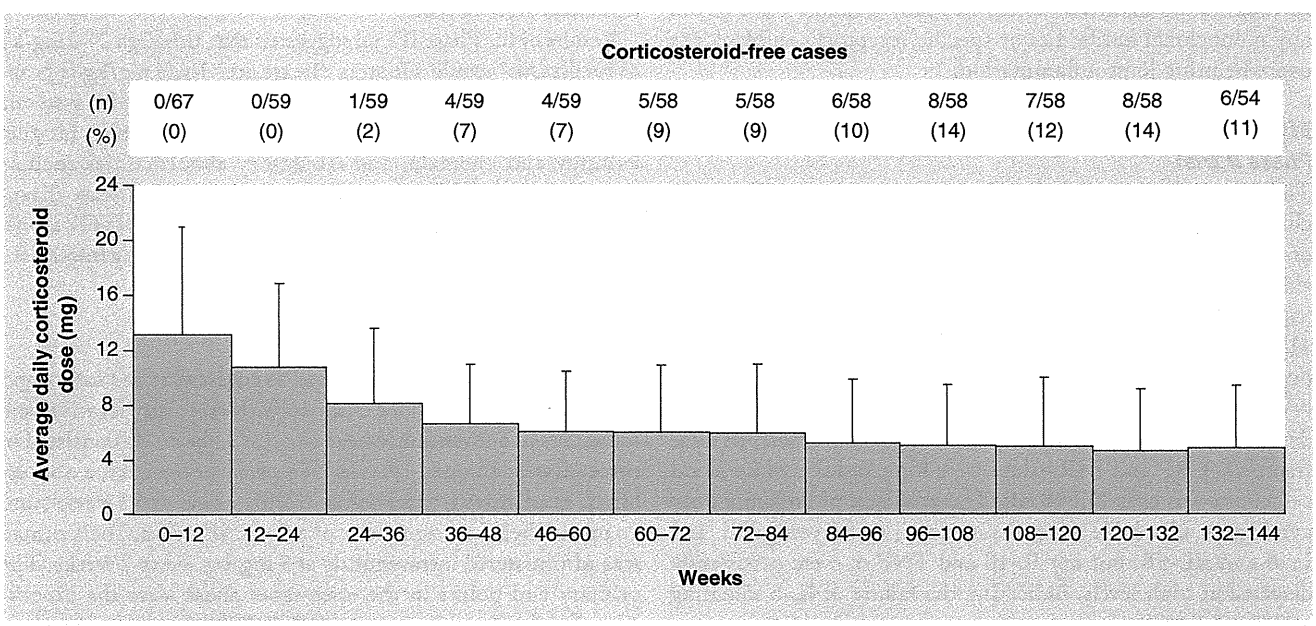

Figure 2. Sparing effects of tocilizumab on corticosteroid therapy. Corticosteroid doses were gradually decreased with repeated tocilizumab administration. The number of corticosteroid-free patients was increased during tocilizumab treatment. Vertical lines represent the standard error bars.

tocilizumab. By week 48 of the open-label extension phase, ACR Pedi 30, 50 and 70% responses were achieved by 98, 94 and 90% of the 48 children, respectively, and improvements in osteoporosis and catch-up growth in children with retarded growth were notably observed. Among the 48 children in the open-label extension study, 69 and 46% were able to reduce corticosteroid doses by at least 30 and 50%, respectively (Figure 2).

Safety & tolerability

No deaths or MAS cases occurred during the entire span of the study and no cases of TB were reported. Two serious adverse events were recorded during the open-label lead-in study: one anaphylactoid reaction and one gastrointestinal hemorrhage from chronic ulceration. Most of the adverse events in the Phase III study were mild or moderate in severity; nasopharyngitis, respiratory tract infection and gastroenteritis were frequently observed, suggesting that owing to inhibition of the IL-6 signaling pathway by tocilizumab, there might have been a potential absence of acute-phase reactions in response to infectious agents. Increases of at least grade 2 ALT and AST were recorded in some patients. Transferases tended to increase early during tocilizumab administration and then subside during continuation of treatment. Mild increases in total cholesterol, mostly within the normal range, were noted. Thus, tocilizumab was safe and well tolerated.

Pharmacodynamics & pharmacokinetics

The Phase II trial revealed that the sufficient dose of tocilizumab was 8 mg/kg at 2-week intervals. Serum concentrations of tocilizumab in the Phase III trial were achieved at steady state during 8–14 weeks after the initial administration and the trough level was 57.4 µg/ml. Children with low body height, light body weight or of young age tended to be those who had rapid disappearance of serum tocilizumab. Since tocilizumab inhibited the IL-6 signaling pathway, CRP could be used as a surrogate marker of inflammation seen in systemic JIA.

Expert commentary

Clinical and laboratory improvement in children with systemic JIA treated with tocilizumab indicates the possible roles played by IL-6 in this inflammatory disease. As described previously, the precise mechanisms of symptoms and signs such as fever, sickness behavior, *CRP* gene expression and chronic inflammatory anemia in relation to proinflammatory cytokines, particularly IL-6, have been revealed, although other manifestations and laboratory changes such as osteoporosis, growth retardation and polyarthritis are likely to be clearly described in the future.

Five-year view

Tocilizumab is the first IL-6-targeted therapy approved for children with systemic JIA in Japan. It modulates the inflammatory process by blocking the IL-6 signaling pathway and is associated with a favorable clinical outcome and safety profile. This provides proof of concept that molecular intervention targeting IL-6R is a viable modality of treatment in systemic JIA as an inflammatory disease.

Treatment of JIA has changed dramatically over the decades. Introduction of weekly methotrexate administered orally has provided remarkable clinical improvement of oligoarthritis and polyarthritis of JIA. For intractable cases, anti-TNF biologic response modifiers, etanercept and adalimumab, have recently emerged as therapeutic options. However, systemic JIA has been left alone behind these therapeutic progresses. The overall efficacy of anti-TNF therapy (etanercept, infliximab and adalimumab) and anti-IL-1 therapy (anakinra) for patients with systemic JIA was reported to be approximately 10% or less [61] and less than half of the patients, respectively [24]. Children with this disease are still under the long-term use of systemic corticosteroids, which inevitably leads to various disorders including iatrogenic Cushing's disease, growth retardation, bone fracture or cataracts. Tocilizumab is effective in children with systemic JIA and is generally safe and well tolerated. It might therefore be a suitable treatment in the control of this disorder, which has so far been difficult to manage. Phase III trials of tocilizumab are now in progress in the EU and the USA. In the near future, tocilizumab will hopefully be available worldwide.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- Tocilizumab has shown clinical improvements in signs and symptoms of systemic juvenile idiopathic arthritis.
- Disease-associated laboratory changes, acute-phase reactant levels, chronic anemia and hypoalbuminemia have been abruptly normalized with tocilizumab treatment.
- Molecular intervention therapy targeting the IL-6 signaling pathway indicated the possible role played by IL-6 in systemic juvenile idiopathic arthritis.
- Although tocilizumab is generally safe and well tolerated, long-term safety data such as data on malignancy and autoimmune diseases are unavailable at this time.