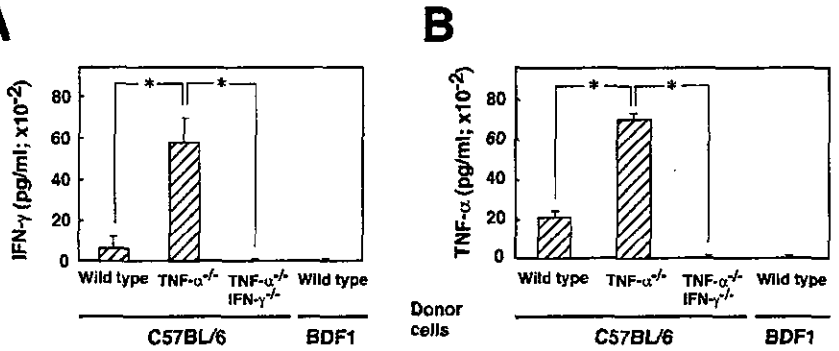

Fig. 4. Requirement of donor-derived IFN-γ for enhanced elevation of serum IFN-γ and TNF-α in mice injected with allogeneic TNF-$\alpha^{-/-}$ spleen cells. BDF1 recipient mice were transferred with syngeneic BDF1, wild-type C57BL/6, TNF-$\alpha^{-/-}$ C57BL/6 or TNF$\alpha^{-/-}$/IFN-$\gamma^{-/-}$ C57BL/6 spleen cells. (A) Serum IFN-γ levels were determined 7 days after GVH induction. (B) Serum TNF-α levels were measured 90 min after LPS injection into GVH mice at 10 days. The data represent means ± SE of three mice. Similar results were obtained in three different experiments. *$P < 0.05$.

Therefore, we tested serum IFN-γ levels in recipient animals. Serum IFN-γ levels in recipient B6D2F1 mice became detectable at 4 days and reached a plateau at 6 days after transfer of wild-type or TNF-$\alpha^{-/-}$ C57BL/6 spleen cells. The kinetics of serum IFN-γ production during GVHD is illustrated in Fig. 3(A). B6D2F1 mice transferred with TNF-$\alpha^{-/-}$ spleen cells showed significantly higher levels of IFN-γ compared with mice transferred with wild-type C57BL/6 spleen cells. Of note, recipient mice treated with IFN-$\gamma^{-/-}$ C57BL/6 spleen cells did not show any increase in serum IFN-γ. This finding indicated that elevation of serum IFN-γ levels in recipient mice is dependent on the capacity of donor cells to produce IFN-γ.

TNF-α decifiency in donor cells enhances TNF-α production by host cells during GVHD

It has been reported that the capacity of host cells to produce TNF-α increases during the development of GVHR (19). Therefore, it was of great interest to determine how TNF-α deficiency in donor cells influences TNF-α production by host cells during GVHR. As shown in Fig. 3(C) and (D), we detected an increase of serum TNF-α levels upon injection of lipopolysaccaride (LPS) in B6D2F1 mice 10 days after treatment with wild-type C57BL/6 splenocytes but not B6D2F1 splenocytes. Recipient mice transferred with TNF-$\alpha^{-/-}$ spleen cells, but not IFN-$\gamma^{-/-}$ spleen cells, showed robust elevation of TNF-α levels in response to LPS injection. Thus, these results demonstrate that defective TNF-α production in donor cells unexpectedly increases host TNF-α production. This phenomenon may contribute to the accelerated deletion of host cells observed in recipient animals that were treated with TNF-$\alpha^{-/-}$ splenocytes.

Suppression of GVHR by donor-derived TNF-α is associated with reduced donor type-1 immunity

As shown in Fig. 3(B) and (D), donor-derived IFN-γ appears to be critical for initiation of early GVHR. To understand the precise role of donor-derived IFN-γ for enhanced IFN-γ and TNF-α production in B6D2F1 mice treated with TNF-$\alpha^{-/-}$ mouse spleen cells, we evaluated TNF-$\alpha^{-/-}$IFN-$\gamma^{-/-}$ splenocyte for induction of GVHD in B6D2F1 mice. Consistent with previous results (Fig. 3), we observed a marked elevation of both serum IFN-γ and TNF-α in recipient mice transferred with TNF-$\alpha^{-/-}$ spleen cells. However, no significant IFN-γ and TNF-α production was observed when animals received splenocytes from TNF-$\alpha^{-/-}$IFN-$\gamma^{-/-}$ mice (Fig. 4). These results suggest that defective TNF-α production by donor cells accelerates GVHR by activating donor IFN-γ-dependent type-1 immunity. To determine which subset of donor T cells is responsible for activating type 1 immunity, we examined the IFN-γ producing capacity of CD4+ and CD8+ T cells from both donor (H-2^{b+}, H-2^{d-}) and recipient (H-2^{d+}) mice by intracellular staining (Fig. 5). In keeping with the results of Fig. 3, B6D2F1 mice treated with TNF-$\alpha^{-/-}$ spleen cells contained a higher frequency of IFN-γ-producing donor-derived CD8+ and CD4+ T cells. Donor-derived CD8+ Tc1 cells were particularly activated to produce IFN-γ (Fig. 5). In addition to the activation of donor Th1 and Tc1 cells, host Th1 and Tc1 cells were also activated to produce IFN-γ. However, when TNF-$\alpha^{-/-}$IFN-$\gamma^{-/-}$ spleen cells were used as donor cells, no significant increase of recipient-derived IFN-γ producing Th1 and Tc1 cells were induced (Fig. 5).

We further demonstrated that anti-host CTL generation, which is essential for the deletion of host cells, is greatly reduced in B6D2F1 mice transferred with TNF-$\alpha^{-/-}$IFN-$\gamma^{-/-}$ spleen cells, as compared with mice transferred with TNF-$\alpha^{-/-}$ spleen cells (Fig. 6). In this experiment, CTL were induced from spleen cells of B6D2F1 mice transferred with donor cells by resensitized with B6D2F1 spleen cells because freshly isolated spleen cells from recipient mice 7 days after donor cell transfer exhibited low levels of cytotoxicity.

Thus, we concluded that in the absence of donor-derived TNF-α during the early phase of GVHR, donor-type-1 immunity, especially Tc1 activity, is activated in a manner that depends on IFN-γ production by donor cells. Subsequently, enhanced host type-1 immunity may induce TNF-α production by host macrophages, which in turn augments GVHR.

Discussion

In the present paper, we clarify the precise role of donor-derived TNF-α in acute GVHD using donor spleen cells from TNF-$\alpha^{-/-}$ mice. We find that the defects in donor-derived TNF-α

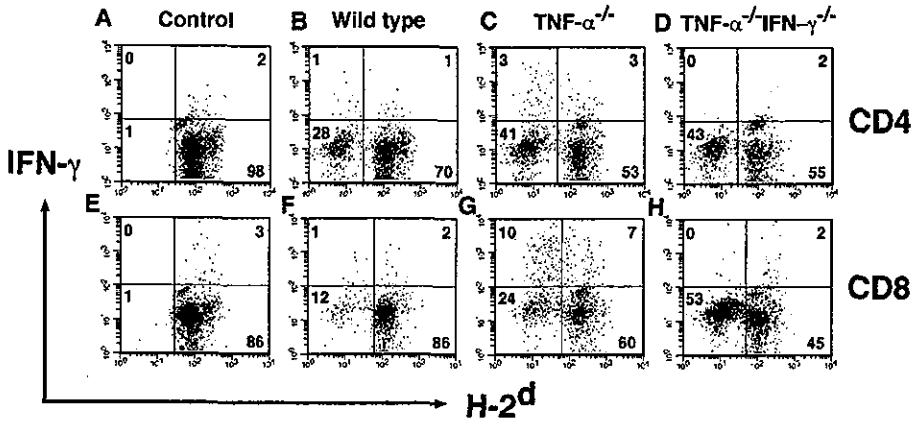

Fig. 5. Induction of donor-derived IFN-γ-producing CD4+ and CD8+ T cells by allogeneic TNF-α$^{-/-}$ spleen cells. BDF1 recipient mice were transferred with syngeneic BDF1 (A and E), wild-type C57BL/6 (B and F), TNF-α$^{-/-}$ C57BL/6 (C and G) or TNF-α$^{-/-}$ IFN-γ$^{-/-}$ spleen cells (D and H). After 7 days, spleen cells were prepared from all mice and their intracellular cytokine expression profile was examined by flow cytometry. (A–D) Expression profile of CD4+ T cells; (E–H) expression profile of CD8+ T cells. *x* and *y* axes indicate H-2^d and IFN-γ expression, respectively. Similar results were obtained in three different experiments.

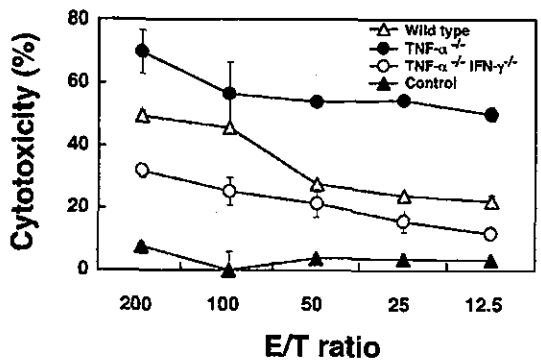

Fig. 6. Requirement of IFN-γ for enhanced generation of anti-host CTL responses in mice treated with allogeneic TNF-α$^{-/-}$ spleen cells. BDF1 recipient mice were transferred with syngeneic BDF1, wild-type C57BL/6, TNF-α$^{-/-}$ C57BL/6 or TNFα$^{-/-}$/IFN-γ$^{-/-}$ C57BL/6 spleen cells. After 7 days, spleen cells obtained from all mice were co-cultured with MMC-treated BDF1 spleen cells and their cytotoxicity against P815 mastocytoma was determined by 4 h ^{51}Cr-release assay. The data represent means ± SE of three mice. Similar results were obtained in three different experiments.

accelerate GVHR, including host IFN-γ and TNF-α production. These data suggest that donor-derived TNF-α may suppress GVHR via controlling donor IFN-γ-dependent type I immunity.

The critical role of proinflammatory cytokines, particularly TNF-α in acute GVHD, has been described in many experimental models and clinical experiments (20–22). The relationship between pre-transplant conditioning regimens, TNF-α production (22–25) and acute GVHD is particularly well-characterized. Chemotherapy and/or total body irradiation damages host tissues including the skin, intestine and liver. Subsequently, the damaged tissues themselves produce TNF-α and LPS, which leak into the systemic circulation and stimulate residual macrophages in the recipient to produce TNF-α. Using TNF-α receptor-deficient mice, it has been demonstrated that host-derived TNF-α plays a critical role in the early activation of allo-reactive donor T cells and increases morbidity and mortality of acute GVHD. In mouse models, anti-TNF-α mAb treatment of lethally irradiated recipients early in bone marrow transplantation reduces mortality and ameliorates pathology in skin and gut lesions. Thus, host-derived TNF-α has been considered to play a critical role in acute GVHD. In our present study we observed paradoxical effects indicating that donor-derived TNF-α suppresses early activation of allo-reactive donor T cells. We found that recipients transferred with TNF-α$^{-/-}$ spleen cells exhibit higher production of IFN-γ by donor Tc1 and Th1 cells, followed by enhanced IFN-γ secretion from residual recipient CD4+ and CD8+ T cells. Activated donor Tc1 and Th1 cells induce host tissue damage by activating host TNF-α-producing macrophages. Thus, TNF-α deficiency in donor cells accelerates the induction of anti-host CTL activity and host TNF-α-producing capacity, which induces severe GVHR. The augmented IFN-γ production is not derived from the different immunological condition between TNF-α-deficient and wild-type mice. TNF-α$^{-/-}$ mice possess the same percentage of immunoregulatory cells (CD4+ Th, CD8+ Tc and B cells) and exhibit the same levels of T cell responses induced by stimulation with anti-CD3 mAb or alloantigen (data not shown).

To explain our finding, several possible mechanisms are considered as follows: (1) TNF-α$^{-/-}$ mice produce higher levels of IFN-γ because they produce less amounts of soluble TNF-α receptor (TNF-αR), which is a blockade for TNF-α, in comparison with wild-type mice; and (2) TNF-α produced by activated T cells or APC has a capability of suppressing hyperactivity of T cells. In terms of soluble TNF-α R production, it was demonstrated TNF-α$^{-/-}$ mice produced the same levels of soluble TNF-α R2 as wild-type mice when they were injected with LPS (data not shown). This result is consistent with previous report that demonstrated the shedding of TNF-α R was induced independently on TNF-α levels (26). Therefore,

it appears to be unlikely that augmented GVHD is derived from the defect of TNF-α R production in TNF-$\alpha^{-/-}$ mice. TNF-$\alpha^{-/-}$ mice exhibit the same levels of T cell responses to anti-CD3 mAb and alloantigen as wild-type mice (data not shown). This observation suggests that TNF-α produced in wild-type mice does not suppress naive T-cell response to antigen. However, recently, we found that TNF-$\alpha^{-/-}$ mice exhibited greatly enhanced T-cell responses in secondary allogeneic responses compared with wild-type mice, if pre-immunized mouse spleen cells were used as responder cells of MLC (data not shown). These findings strongly suggest that TNF-α may act as a negative feedback factor for T-cell hyperactivity as IL-27 does (27).

As classical risk factors of acute GVHD, HLA mismatches between donor and recipient are well-documented (28,29). Many studies have examined risk factors by studying the production of inflammatory cytokines. Holler *et al.* (30) have demonstrated that host-related secretion of TNF-α during pretransplant conditioning correlates with subsequent GVHD and mortality after transplantation. Analysis of clinical risk factors for enhanced TNF-α response suggested a role for endogenous endotoxin and immunogenetic factors of cytokine activation. In human, the gene encoding TNF-α is located within the MHC locus on chromosome 6 (31), and the inducibility of TNF-α has been associated with certain HLA-class II genotypes (32). A close association between cytokine gene polymorphisms and cytokine inducibility has also been identified. There is a single base polymorphism in the TNF-α gene at position –308(G/A) (33). The rare allele TNF2(A) is closely associated with HLA A1, B8 and DR3 (34). When allelic distribution of this polymorphism was analyzed in 72 BMT recipients, a clear association of the TNF2(A) allele with enhanced *in vitro* TNF-α production in response to LPS and subsequent development of acute GVHD was noted (35). Another study has reported that the d3 homozygous allele of the TNF-α microsatellite is preferentially associated with grade III/IV GVHD (36). While these data indicate a role for TNF-α production by the recipient in predicting the development of GVHD, the relationship between donor TNF-α production and GVHD severity has never been elucidated. In a mouse model, Cooke *et al.* (37) have reported that LPS responsiveness of donor accessory cells correlates with GVHD severity. These investigators suggested that TNF-α production by LPS-stimulated donor cells may be a risk factor for the development of donor T cell responses to host antigens. In contrast, our data indicate that donor-derived TNF-α has a suppressive effect on the initiation of GVHR. The discrepancy in these results may be due to different cellular sources for TNF-α production. Cooke *et al.* (37) investigated TNF-α producing capacity of LPS-reactive TLR4-bearing APC populations using C3H/HeJ TLR4-deficient mice, while we assessed TNF-α producing ability of donor T cells in addition to APC. We found that accelerated GVHR induced by transfer with TNF-$\alpha^{-/-}$ spleen cells was abrogated when either $CD4^+$ T cells or $CD8^+$ T cells were eliminated from donor spleen cells (data not shown). This result indicates a critical role of T cells in the initiation of GVHR, which might be triggered independently of donor APC populations. Our finding that (i) donor TNF-$\alpha^{-/-}$IFN-$\gamma^{-/-}$ cells do not accelerate GVHD (Figs 4–6) and (ii) donor-derived $CD8^+$ T cells are major IFN-γ-producing T cells during the early stage of GVHR (Fig. 6), strongly suggests that memory-type IFN-γ producing $CD8^+$ T cells play a critical role in GVHR induction. We are currently investigating the detailed cellular mechanisms by which donor-derived TNF-α suppressed donor type-1 immunity.

Donor lymphocyte infusion (DLI) has been frequently utilized for treatment of recurrent hematologic malignancies after allo-HST (38). This procedure re-induces complete remission in many patients, but the risk of lethal GVHD is still hard to predict. Because DLI is performed after primary allo-HST, TNF-α production by damaged tissues in the host after conditioning therapy is not as high as compared with donor-derived TNF-α. At present, it may be of great importance to investigate whether severity of GVHD can be predicted by examining donor TNF-α producing capacity, especially by T cells. We are currently investigating this issue.

In conclusion, we have shown, for the first time, that TNF-$\alpha^{-/-}$ donor cells accelerate GVHR early after allogeneic transplantation. Therefore, donor-derived TNF-α may suppress GVHD morbidity. These findings provide novel approaches to detect patients undergoing allo-HST with increased risk for GVHD. Further studies will be required to determine the role of donor-derived TNF-α in a clinical transplantation setting.

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Abbreviations

DLI	donor lymphocyte infusion
GVHD	graft versus host disease
GVHR	graft versus host reaction
HST	hematopoietic stem cell transplantation

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6．自己免疫疾患に対する造血幹細胞移植

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Summary　自己免疫疾患の生命予後は，免疫抑制療法や支持療法の進歩により一般的に良好であるが，一部の症例は，従来の治療に抵抗し，機能障害や臓器障害が急激に進行する。近年，このような難治性自己免疫疾患に対して，自家造血幹細胞移植を併用した超大量免疫抑制療法が試みられている。現時点で，およそ650例が全世界で施行されており，その有効性が明らかになりつつある。一方で，共通治療プロトコールの確立，症例の選択基準，治療関連死の克服，移植後の易感染性など今後の課題も多い。

はじめに

自己免疫疾患は遺伝要因を背景に，喫煙，感染，性ホルモンなどの環境要因が作用して自己反応性リンパ球が出現し，直接またはBリンパ球を介した自己抗体産生促進により組織障害を引き起こす[1)]。生命予後は，最近の免疫抑制療法や支持療法の進歩により一般に良好であるが，一部の症例は，従来の治療に抵抗し急激な機能障害や臓器障害が進行して日常生活の質(quality of life:QOL)が著明に低下する。また，このような重症例では生存期間の明らかな減少が認められる[2,3)]。近年，このような難治性自己免疫疾患に対して，自己反応性リンパ球の除去を目的とする自家造血幹細胞移植(autologous hematopoietic stem cell transplantation：AHSCT)を併用した超大量免疫抑制療法(図1)が試みられ，その有用性が報告されてきている[4~7)]。現時点でおよそ650例が全世界で施行されている[5)]。本総説では，自己免疫疾患に対する造血幹細胞移植の適応疾患，治療法および成績について最近の知見を紹介する。

1．自己免疫疾患に対するAHSCTの理論的背景

自己免疫疾患発症動物モデルに同種骨髄移植を

《略語一覧》
QOL (quality of life；日常生活の質)
AHSCT (autologous hematopoietic stem cell transplantation；自家造血幹細胞移植)

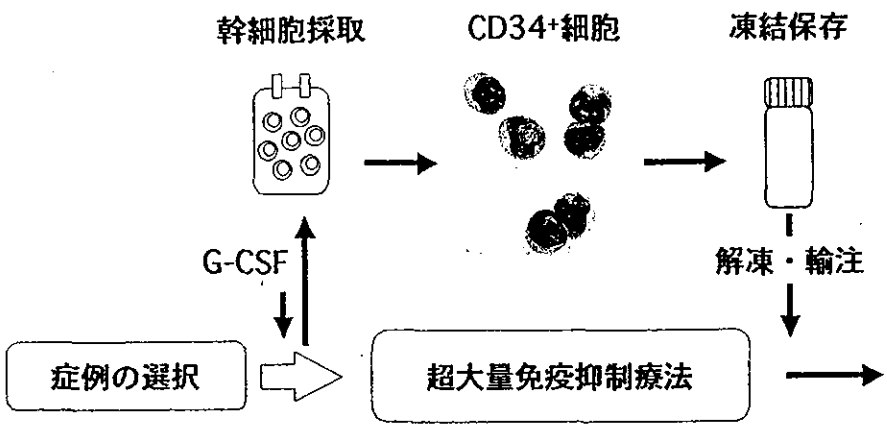

図1　自己免疫疾患に対する自家末梢血幹細胞移植を併用した超大量免疫抑制療法
適格症例を厳密に選択したのち，患者に G-CSF を投与し末梢血に造血幹細胞を動員して採取し，凍結保存する。あらかじめ cyclophosphamide を投与し，骨髄回復期に G-CSF の投与を行う方法も頻用されている。CD34⁺細胞として純化することによって，移植片から自己反応性リンパ球を除去する試みも行われている（*ex vivo* purging）。移植前処置として超大量免疫抑制療法を施行して自己反応性リンパ球を除去し（*in vivo* purging），移植細胞を解凍・輸注する。

行い病態の改善が得られたこと，血液疾患に自己免疫疾患を合併した症例で，同種移植後に白血病と自己免疫疾患ともに寛解した報告が蓄積されたことが，自己免疫疾患に対する AHSCT の理論的背景となった[1]。同種移植では，移植造血幹細胞自体に異常がなく，自己免疫疾患を治癒させる可能性のある有望な方法とも考えられている[1]。しかし，移植関連死亡率(transplant-related motality：TRM)が 15～30%と高い。一方，AHSCT では TRM が 5%と低く，より安全な方法として全世界の多くの臨床治験で採用されている[1]。

自家移植による自己免疫疾患治療の有効性の機序は，①自己反応性リンパ球の根絶による異常免疫機序の破壊，②免疫システムの再構築と新しく産生されたリンパ球の自己組織に対する免疫寛容の獲得，が推定されている。しかし，同療法後に再燃する症例もしばしば認められ，その機序は①自己反応性リンパ球の再輸注や体内残存，②自己抗原の再暴露，によるものと考えられる[1]（図2）。自己反応性リンパ球の除去を目的に，造血幹・前駆細胞（CD34⁺細胞）の純化移植やリンパ球除去移植が行われている[1, 5, 6]。この場合，免疫再構築が遷延するため日和見感染症やヘルペスウイルス属の再活性化に注意を要する[6]。

2．移植適応疾患

移植の適応症例は，従来の治療に抵抗性で，その疾患によって生命が脅かされる予後不良な状態，ないしはその疾患自体はコントロール可能でも機能障害により QOL が著明に低下する可能性がある場合である。また，移植の障害となる不可逆性臓器障害がまだ生じていない症例であると考えられている[1~5]。現在まで，多くの自己免疫疾患に対して AHSCT が施行されているが，中でも全身性硬化症（systemic sclerosis：SSc），全身性

《略語一覧》
TRM（transplant-related motality；移植関連死亡率）　SSc（systemic sclerosis；全身性硬化症）

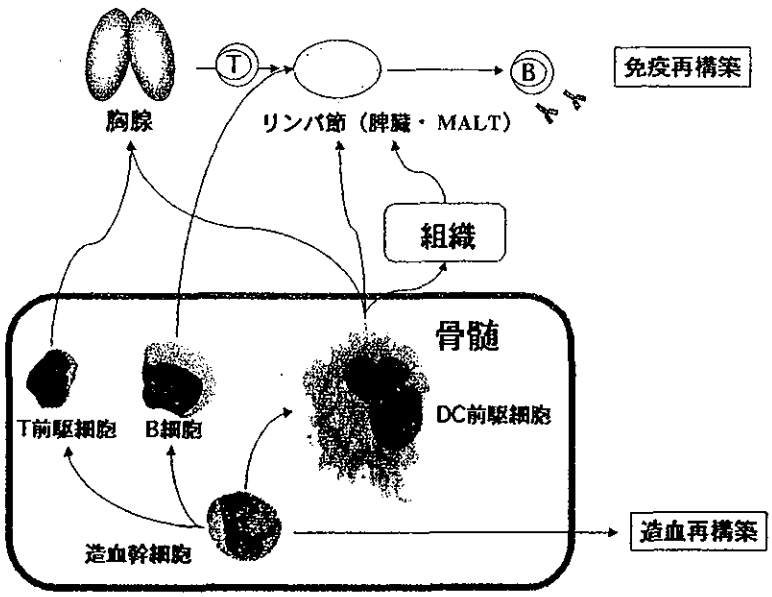

図2　純化CD34細胞移植後の造血および免疫再構築

移植された造血幹細胞より分化した，あるいは移植片中に含まれている骨髄系前駆細胞とリンパ系前駆細胞より造血とリンパ系再構築がおこる。造血幹細胞に由来する樹状前駆細胞は胸腺，リンパ節，脾臓，mucosa-associated lymphoid tissue（MALT）および皮膚などの組織中で樹状細胞に分化する[10]。自己免疫疾患における自家移植の意義として，①自己反応性リンパ球の根絶による異常免疫機序の破壊，②免疫システムの再構築と新しく産生されたリンパ球の自己組織に対する免疫寛容の獲得，が推定されている。これに対し，再燃の機序として，①自己反応性リンパ球の再輸注や体内残存，②自己抗原の再暴露，が推定されている。

エリテマトーデス（systemic lupus erythematodes：SLE），関節リウマチ（rheumatoid arthritis：RA），若年性関節リウマチ（juvenile rheumatoid arthritis：JRA）および多発性硬化症（multiple sclerosis：MS）の症例数が多い[5]。本邦での移植適応基準に関しては，海外の適応基準を参考にした北海道大学第二内科の移植適応基準[8]があり著者等の施設もこれに準じている。

3．幹細胞動員と移植前処置

末梢血からの造血幹細胞の動員方法としては顆粒球コロニー刺激因子（granulocyte-colony-stimulating factor：G-CSF）単独もしくはシクロ

《略語一覧》
SLE（systemic lupus erythematodes；全身性エリテマトーデス）
RA（rheumatoid arthritis；関節リウマチ）　JRA（juvenile rheumatoid arthritis；若年性関節リウマチ）
MS（multiple sclerosis；多発性硬化症）
G-CSF（granulocyte-colony-stimulating factor；顆粒球コロニー刺激因子）
CY（cyclophosphamide；シクロホスファミド）

ホスファミド（cyclophosphamide：CY）2～4 g/m²の併用がある。G-CSF単独の動員では，原病の増悪やマクロファージ活性化症候群を呈した症例の報告もあり注意を要する。目標採取細胞数は2～5×10⁶ CD34⁺細胞/kgである。CD34⁺細胞純化により動員細胞からのTリンパ球除去が行われている場合も多い[5,6]。移植前処置は①CY（200 mg/kg）±抗胸腺細胞グロブリン（anti-thymocyte globulin：ATG），②全身放射線照射（total body irradiation：TBI）（800 cGy）±CY（120 mg/kg），③CY＋ブスルファン（busurufan：BU），④BEAM（carmustine, etoposide, cytosine arabinoside, melphalan），⑤TBI（800 cGy），CY（120 mg/kg），ATG（90 mg/kg），などが使用されている[5~7]。BEAMは骨髄破壊的前処置であるが，薬剤の髄液移行の点から主にMSに対して用いられている。ATGやTBIの必要性も含め標準的レジメは確立していないが，TBIは合併症が多いため行われなくなってきている。

4．移植成績

全世界で650症例以上の自己免疫疾患患者で造血幹細胞移植が行われている[5]。その中心はヨーロッパであるが，European Blood and Marrow Transplant Group（EBMT）とEuropean League against Rhuematism（EULAR）でも患者の選択，動員法，purgingや前処置についての共通基準はない。したがって，治療反応性や再発率の評価は複雑であり，その結果の解釈には注意が必要である。2000年の米国血液学会で発表された198例の移植成績[9]を図3に示した。興味深いことは，RAを例外として，改善や症状の安定が見られたほとんどの症例が現在もなお，良好なQOLを保っていることである。以下，主要な疾患の最近の成績を紹介する。

1）SSc

SScは，全身の臓器に線維化を生じる原因不明の疾患であり，その内臓病変の有無が生命予後に関連してくる。皮膚硬化と臓器障害は発症から数年間で完成されることが多く，SScの経過（生存期間の延長，または臓器障害の予防や進行速度の遅延）を変えることができる最良の機会は最初の3～4年に限られる。以上からAHSCTの適応となるSScは，発症3～4年未満で心，肺，腎などに軽度の臓器障害を有する患者であると考えられている。びまん性進行性やびまん性肺病変を有するSSc症例の5年生存率は30%～70%と不良である[10]。

SScでは，平均12カ月経過観察された41例中28例（69%）でskin scoreが25%以上改善したとEBMT/EULARから報告された[11]。また，米国からは，19例中15例でskin scoreが25%以上改善したと報告された[10]。著者（澤田）が以前在籍していた北海道大学二内科（小池隆夫教授）でも3例のSScに対してCD34⁺細胞移植を併用した超大量免疫抑制療法を施行した。移植後一年の時点でskin scoreが25%以上改善し経過良好である。また，当科（秋田大学）で最近経験した1例についても移植後3カ月の時点で25%のskin scoreの改善が得られている。現在，第三相臨床試験（autologous stem cell transplantation interna-

《略語一覧》
ATG（anti-thymocyte globulin；抗胸腺細胞グロブリン）　TBI（total body irradiation；全身放射線照射）
BU（busurufan；ブスルファン）
BEAM（carmustine, etoposide, cytosine arabinoside, melphalan）
EBMT（European Blood and Marrow Transplant Group）　EULAR（European League against Rhuematism）
ASTIS trial（autologous stem cell transplantation international scleroderma；第三相臨床試験）

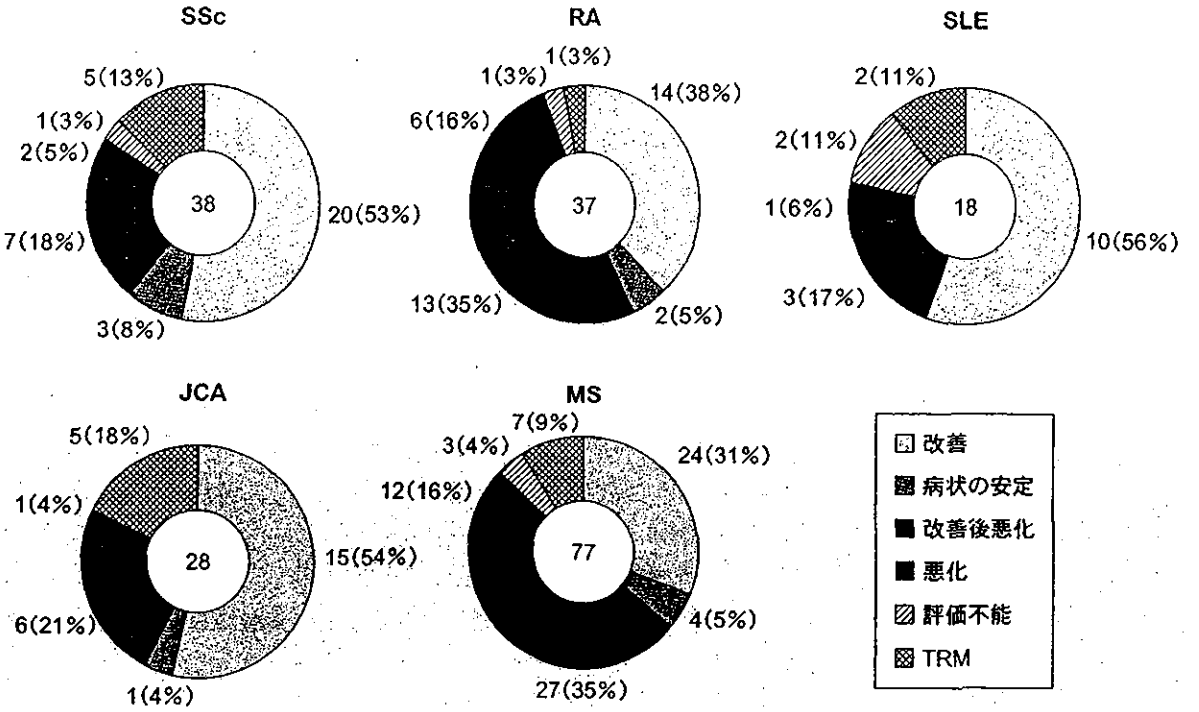

図3　自己免疫疾患に対する自家造血幹細胞移植の成績

SSc：全身性硬化症，RA：関節リウマチ，SLE：全身性エリテマトーデス，JRA：若年性関節リウマチ，MS：多発性硬化症，TRM：治療関連死　（文献9を改変）

tional scleroderma：ASTIS trial）がヨーロッパで進行中であり，米国でも今後予定されている[11]。

SScにおいて注意すべきことは，心臓が何らかの障害を受けていることが多く，心筋や伝導系に，微小血管の障害に基づく斑状の線維化を認めることである[12]。しばしば無症状であるが多くの症例で死因となりうる。EBMT/EULARでは59例（12%）のTRMが報告されている。うちSScの症例は19例（32%）で，死因を同定できた症例のうち，心臓関連死は5例（SScのTRMのなかで26%）であった。このうちCYによる毒性が疑われたのは2例である。移植前の心機能のスクリーニング検査で異常は認められなかった[13]。我々が経験した症例は，頻脈と脳ナトリウム利尿ペプチド（brain natriuretic peptide：BNP）の軽度の上昇を認め，CY（2 g/m²/日，2日間）による幹細胞動員時に心不全を発症した。そこで移植前処置ではCYを従来の（50 mg/kg/日，4日間投与）半量に減量し（50 mg/kg/日，2日間投与），心毒性の少ないthiothepa（TT：5 mg/kg，1日2回投与，1日間）を併用したTT-CY療法を用いた。このようなTT-CY療法は，特に心予備能の低下が疑われる症例に対し，心毒性の軽減のために有効な方法であると考えられる[14, 15]。

2）RA

RAは関節炎の寛解，再燃を繰り返す例が多い

《略語一覧》
BNP（brain natriuretic peptide；脳ナトリウム利尿ペプチド）
TT（thiothepa）

が，幅広いスペクトラムを持ちその予後の予測は難しい。最近の米国の調査で RA の生命予後はかなり悪いとの見解[16]もあるが，軽症の例ほど研究から脱落しやすいなどの問題点もありいまだ議論のあるところである。RA における移植適応はそれゆえ，関節破壊による QOL の低下をいかに阻止するかという観点から捉えられる。

EBMT/EULAR によって 73 例の自家移植例が集積された[1]。平均年齢 43 歳，74%が女性で，86%がリウマチ因子陽性であった。Health Assessment Questionnaire (HAQ) score は平均 1.4 と重度の機能障害を有する症例が大部分を占めていた。幹細胞動員による flare (症状増悪) は，評価可能症例 56 例中 8 例 (14%) に生じ，動員前処置による相違は CY + G-CSF (±その他) と G-CSF 単独群間で認められていない。ほとんどの症例 (59/73 例：81%) が移植前処置として CY 総量 200 mg/kg の投与を受けた。TRM は認められなかった。16 カ月の平均観察期間のある時期に，49 例 (67%) においてアメリカ・リウマチ学会会議 (American College of Rheumatology：ACR) criteria で 50%以上の症状改善が認められたが，ほとんどの症例 (58/63 例：92%) で再燃した。疾患修飾性抗リウマチ薬 (disease modifying anti-rheumatic drugs：DMARDs) の再投与が確認できた 43 症例中，21 例 (49%) では移植前の状態よりも改善，22 例 (51%) では同等か悪化であった。自家移植によって DMRADs に対する反応性が改善される可能性が指摘されている。

移植の効果予測因子は唯一，RF 陰性であった。しかし，単一施設の検討では RF の有無は移植反応性に関与しないとの報告もあり，今後の検討を要する。以上の 73 症例中，抗 TNFα 一抗体が使用されたのは 4 例のみであった。現在，難治性 RA に対して抗サイトカイン療法が可能になり，RA の予後は著明に改善されてきた。しかし，症例によっては病勢のコントロールが不十分で新規薬剤によっても治癒は望めない。今後はそのような抗サイトカイン療法無効症例に AHSCT が選択されると考えられる[1]。

3) SLE

SLE の生命予後はステロイドの登場で劇的に改善した。ただし難治性で生命予後に直接かかわる病態もある。一方，慢性の腎症，皮膚症状，関節症状や，長期ステロイド使用による骨病変，動脈硬化性病変のため QOL が著しく制限される患者も多い。

EBMT/EULAR によって 51 例が集積された。登録症例のほとんどが，ループス腎炎や CNS ループスを有していた[1]。解析可能 48 例中 27 例 (56%) が改善，14 例 (29%) は改善後に再発，7 例 (15%) が死亡した。死亡 7 例中 5 例が TRM であった (5/48 例：10.4%)。その他，複数の報告を合計した 23 症例では，CY (200 mg/kg) と ATG (90 mg/kg) などの前処置後に全例で疾患活動性の改善が認められた[17~20]。しかし，その後 8 例で免疫抑制療法が必要になっている。今後，第三相臨床試験が欧米で予定されている (BMT)。

《略語一覧》
HAQ (Health Assessment Questionnaire)
ACR (American College of Rheumatology；アメリカ・リウマチ学会会議)
DMARDs (disease modifying anti-rheumatic drugs；疾患修飾性抗リウマチ薬)
TNF (tumor necrosis factor；腫瘍壊死因子)
GOT (glutamic axaloacetic transaminase；グルタミンオキザロ酢酸トランスアミナーゼ)
GPT (glutamic pyruvic transaminase；グルタミン・ピルビン酸トランスアミナーゼ)
LDH (lactic acid dehydrogenase；乳酸脱水素酵素)
MAS (macrophage activating syndrome；マクロファージ活性症候群)

4）JRA

JRAの全身型のうち，早期から関節炎がみられる症例の多くは関節破壊の進行が比較的早く関節炎としての予後は悪い。また経過中，特に発熱，リンパ節腫脹，肝脾腫とともに血小板減少，白血球減少，貧血，GOT，GPT，LDHの上昇，低フィブリノゲン血症，骨髄での血球貪食が認められる場合がある。マクロファージ活性症候群（macrophage activating syndrome：MAS）とよばれ，T細胞，マクロファージの異常活性化による高サイトカイン血症に基づく症候群である。治療はいかに早期に活動性を抑制し，関節破壊まで炎症を進ませないかという点につきる。積極的な治療を行っても反応せず活動性であるという証拠，または治療を受容できない毒性がある場合が移植適応であると考えられる[21]。

難治性JRAでの改善率は35例中27例（77%）であったが，その後7例が悪化している[4]。本邦ではKishimotoらによって3例のJRAに対するAHSCTが報告されている[22]。うち2例で完全寛解が得られた。JRAではMASによる死亡例も報告されており注意を要する[1]。AHSCT後のMASの発症には，移植片からのT細胞の除去が関与すると推定され，制御性T細胞の欠如によってマクロファージが活性化され血球貪食症候群を引き起こす機序が想定されている。予防には，ステロイドで制御できない活動性（発熱など）を有する患者は移植適応外とすることが勧告されている。治療として，説明困難な39℃以上の発熱が2日間以上続く場合にはMASを考慮してmethylprednisoloneとcyclosporinの併用が推奨されている[23]。JRAの予後は抗TNF抗体などの新規薬剤によって改善される可能性があり，今後の適応はそのような薬剤に反応しない症例に限定されると思われる。

5）MS

自己免疫疾患には膠原病のみではなく神経疾患の一つであるMSも含まれる。IFN-betaやcopaxone療法によって，MSの発作数は30～50%減少する。しかし，およそ2/3の患者が再発と寛解を繰り返す経過をとり，進行性MSの症例は機能障害が進行するとともに早期に死亡する。これらの患者は疼痛と運動障害のために生きていくことが耐え難くもなる。

MS全体での改善率は95例中35例（37%）であるが，その後4例が悪化している。しかし，症状安定が26例に認められている[1]。したがって，少なくとも病状安定化には有効と考えられる。

おわりに

AHSCT併用超大量免疫抑制療法は，自己免疫疾患の長期的寛解が期待される治療法であるが，無効例が存在することや治療関連死が依然5～9%と高率であるなどの問題点もある[1,5]。今後，適応基準と個々の疾患に対応した移植前処置の検討が必要である。さらに，長期間の経過観察や大規模臨床試験により，疾患改善度，強力な免疫抑制の副作用の評価，超大量化学療法の長期的影響を明らかにし，本治療法の安全性向上と有用性の検証を行うことが必要と考えられる。

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Polymorphisms in the thymidylate synthase and methylenetetrahydrofolate reductase genes and sensitivity to the low-dose methotrexate therapy in patients with rheumatoid arthritis

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Abstract. Methotrexate (MTX), widely used in the treatment of rheumatoid arthritis (RA), inhibits dihydrofolate reductase (DHFR) and folate-dependent enzymes. Thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR) are key enzymes in the folate metabolism and both have been shown to be polymorphic affecting the enzyme activity. To clarify the association between these genetic variations and MTX-related toxicity and efficacy in the treatment of RA, a total of 167 Japanese individuals with RA, including 52 and 63 patients treated with low-dose MTX with or without adverse effects, respectively, and 52 patients without MTX administration were analyzed. Among the 93 patients treated with MTX for >2 months, significantly more patients homozygous for the triple-repeat allele of the polymorphism in the promoter region of the *TYMS* gene required higher dose of MTX compared to those having at least a double-repeat allele (P=0.033). The incidence of ≥50% improvement in the serum CRP level was significantly higher in patients homozygous for the deletion allele of the polymorphism in the 3'-untranslated region (UTR) of the *TYMS* gene (P=0.0383). The allele frequency of the insertion/deletion polymorphism in the *TYMS* 3'UTR in Japanese was significantly different from that in Caucasians (P<0.0001), as was the tandem-repeat polymorphism in its promoter region. On the other hand, *MTHFR C677T* and *A1298C* polymorphisms showed no association with MTX-related toxicity or efficacy. Our results suggest that the genotyping for the *TYMS* polymorphisms may become a useful indicator in determining the appropriate dose of MTX in patients with RA.

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Introduction

Methotrexate (MTX) is widely used in patients with collagen vascular diseases as well as with malignancies, expecting anti-inflammatory, anti-proliferative, and immunosuppressive effects whose mechanism of action is not fully established. Especially in the treatment of rheumatoid arthritis (RA), MTX has been proved to be one of the most effective and rapidly working drugs (1). MTX is a structural analog of folic acid, thus competitively inhibits dihydrofolate reductase (DHFR), which is essential for reduction of the dihydrofolate to tetrahydrofolate (THF). Therefore, oral administration of MTX results in a depletion of THF, which is the precursor of bioactive folate cofactor forms required for synthesis of thymidylate, purines, methionine, and serine (2). Cells exposed to MTX are presumed to die due to the depletion of reduced folates.

MTX is also known to inhibit other folate-dependent enzymes besides DHFR, thymidylate synthase (TS), methylene tetrahydrofolate reductase (MTHFR), and others (2). TS is an enzyme that catalyzes the conversion of deoxyuridylate to deoxythymidylate, which is essential for DNA synthesis and repair. The promoter enhancer region of the *TYMS*, the gene encoding TS, was shown to be polymorphic, containing either two or three 28-bp tandem-repeat sequences (3). The presence of the triple versus double 28-bp repeat was shown to enhance the TS expression. In addition, a 6-bp deletion polymorphism at bp 1494 in the 3'-untranslated region (UTR) of the *TYMS* gene was identified (4). The potential effects of this polymorphism on the enzyme activity have not been defined yet. MTHFR is one of the regulating enzymes in the remethylation of homocysteine from methionine. Two common genetic polymorphisms have been reported in the *MTHFR* gene, the *C677T* and the *A1298C* single nucleotide substitutions that lead to altered amino acids. The *C677T* substitution alone or combination of them were reported to decrease the MTHFR enzyme activity and increase the plasma homocysteine levels (5), and RA patients with the *C677T* variation had a greater increase of homocystein during treatment of MTX (6).

The mechanism of the action of high-dose MTX therapy, widely used in cancer chemotherapy, is thought mainly

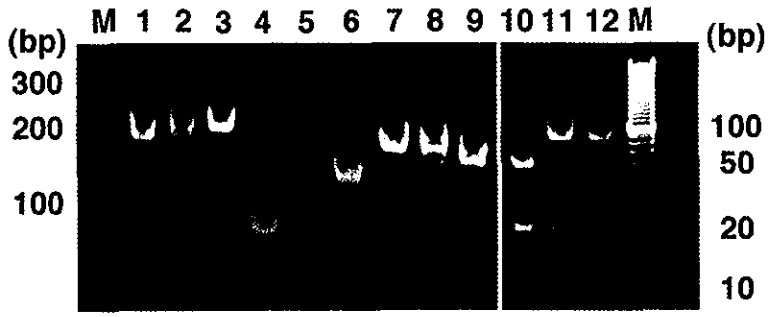

Figure 1. PCR-based genotyping for the *TYMS* and the *MTHFR* polymorphisms. Lane M, marker DNA; lanes 1-3, PCR products of the *TYMS* promoter region (lane 1, 2R/2R; lane 2, 2R/3R; and lane 3, 3R/3R genotype); lanes 4-6, PCR products of the *TYMS* 3'UTR digested with *Dra*I (lane 4, 6 bp/6 bp; lane 5, 6 bp/0 bp; and lane 6, 0 bp/0 bp); lanes 7-9, PCR products containing the *MTHFR C677T* polymorphism digested with *Hin*fI (lane 7, C/C; lane 8, C/T; and lane 9, T/T); lanes 10-12, PCR products containing the *MTHFR A1298C* polymorphism digested with *Mbo*II (lane 10, A/A; lane 11, A/C; and lane 12, C/C).

due to its inhibitory effects on the DHFR and TS activities necessary for DNA synthesis and cell proliferation. However, the anti-proliferative effect cannot explain the full spectrum of action of low-dose MTX in the treatment of RA. Currently, it was speculated that the beneficial clinical effects of low-dose MTX are the results of anti-inflammatory action possibly mediated by adenosine, whereas the toxicity may be more complicated (1). Irrespective of its well-documented efficiency of MTX, about 30% of RA patients who received MTX lead to discontinuation within one year mainly due to toxicity (7). Although a few possible predictors of the toxicity, such as baseline white blood cell counts and creatinine levels have been reported (8), neither the efficacy nor the toxicity during MTX therapy is individually predictable.

The present study was performed to examine whether any genotypes of the *TYMS* or the *MTHFR* polymorphisms are associated with the development of efficacy or toxicity of low-dose MTX therapy in patients with RA.

Materials and methods

Patients. A total of 167 patients with RA (135 females and 32 males) were enrolled in this study, including 115 patients with a history of MTX administration (MTX group) and 52 patients without it (non-MTX group). All patients had been diagnosed as having RA according to the 1987 American College of Rheumatology (formerly, the American Rheumatism Association) criteria (9). Clinical information on the patients was obtained from the chart review. Functional class and anatomic grading were determined according to the criteria of Steinblocker (scales 1-4). All patients in the MTX group underwent clinical and laboratory assessments, including drug-related toxicity and blood tests such as blood cell counts, serum levels of C-reactive protein (CRP), liver-enzymes, alkaline phosphatase, total bilirubin, albumin, and creatinine every 1-3 months. This study was approved by the Institutional Ethics Committee and written informed consent was obtained from all patients.

Evaluation of the toxicity of MTX. The adverse effects of MTX were assessed by the medical records. We defined the bone marrow toxicity when the white blood cell count fell below 3,500/mm^3 and 75% of that before administration of MTX. We defined hepatotoxicity when the serum level of alanine aminotransferase (ALT) elevated over the range of normal levels after the initiation of MTX therapy or increase in MTX dosage.

Evaluation of the efficacy of MTX. To assess the effects of MTX in the MTX group, serum levels of CRP were compared before and after the MTX therapy. The CRP level after the MTX therapy was evaluated at least more than two months after the commencement of the MTX therapy, and patients who discontinued MTX or decreased its dose within two months due to adverse effects were excluded in the evaluation of efficacy. During the MTX therapy, concomitant drugs, such as corticosteroid, additional disease modifying antirheumatic drugs (DMARDs), and non-steroidal anti-inflammatory drugs (NSAIDs), were maintained on a stable dose in all patients at least for one month before the measurement of CRP value. To assess the genetic association with the sensitivity to MTX, patients in the MTX group were divided into two subgroups according to the MTX dosage, <6 mg/week and ≥6 mg/week, and the correlation between the maintenance dose of MTX required and the genotypes for each polymorphism was investigated.

Genotype analysis. Genotypes of a 28-bp tandem-repeat polymorphism in the promoter region and a 6-bp deletion polymorphism in the 3'UTR of the *TYMS* gene and those of the *C677T* and *A1298C* polymorphisms of the *MTHFR* gene were determined using the polymerase chain reaction (PCR) or PCR-restriction fragment length polymorphism (RFLP) method. The primers were synthesized according to previous reports (3,4,10,11) with slight modifications as follows: *TYMS* promoter sense, 5'-GTGGCTCCTGCGTTTC CCCC-3'; antisense, 5'-TCCGAGCCGGCCACAGGCAT-3'. *TYMS* 3'UTR sense, 5'-CAAATCTGAGGGAGCTGAGT-3'; antisense 5'-CAGATAAGTGGCAGTACAGA-3'. *MTHFR C677T* sense, 5'-TGAAGGAGAAGGTGTCTGCGGGA-3'; antisense, 5'-AGGACGGTGCGGTGAGAGTG-3'. *MTHFR A1298C* sense, 5'-CTTTGGGGAGCTGAAGGACTACTAC-3'; antisense, 5'-CACTTTGTGACCATTCCGGTTTG-3'. After PCR amplification, the allele containing two 28-bp repeats (2R) in the *TYMS* promoter region is expected to yield 212-bp fragments, while that containing three repeats (3R) yields 240-bp fragments. For the 6-bp insertion/deletion polymorphism in the *TYMS* 3'UTR, *Dra*I digestion produces 70- and 88-bp fragments in the wild insertion allele (6 bp)

Table I. Demographic and clinical features of 115 patients with rheumatoid arthritis treated with MTX (MTX group).

	Adverse effects	
	Present (n=52)	Absent (n=63)
Sex		
No. of females	42 (81%)	53 (84%)
Age		
Mean ± SD (years)	59.7±9.8	60.0±11.7
Range (years)	35-79	29-80
Disease duration		
Mean ± SD (years)	10.8±7.3	11.8±8.2
Range (years)	1-33	0.9-37
Functional class		
Mean ± SD	2.2±0.6	2.0±0.5
Anatomic grade		
Mean ± SD	2.5±0.9	2.5±0.8
RF		
Positive patients	40 (77%)	39 (62%)
CRP		
Mean ± SD (mg/dl)	1.9±2.2	1.6±1.8
ESR		
Mean ± SD (mm/hour)	44.8±28.5	42.4±27.2
Dose of MTX		
Mean ± SD (mg/week)	5.7±2.3	5.3±1.6
Range (mg/week)	2-12	2-10
Folate supplementation		
No. of patients	21 (40%)[a]	11 (17%)
Additional drugs		
No. of patients		
Corticosteroids	43 (83%)	56 (89%)
DMARDs	16 (31%)	20 (32%)
NSAIDs	36 (69%)	46 (73%)

RF, rheumatoid factor; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DMARDs, disease-modifying antirheumatic drugs; NSAIDs, non-steroidal anti-inflammatory drugs; functional class and anatomic grading were determined as described in Materials and methods. [a]P=0.0063.

and an uncleaved 152-bp fragment in the deletion allele (0 bp). For the *MTHFR C677T* polymorphism, *Hin*fI digestion produces an uncleaved 198-bp fragment in the wild allele (C) and 175- and 23-bp fragments in the *677T* variant allele. For the *MTHFR A1298C* polymorphism, *Mbo*II digestion produces 56-, 31-, 30-, 28-, and 18-bp fragments in the wild allele (A) and 84-, 31-, 30-, and 18-bp fragments in the *1298C* variant allele. These digested and/or undigested PCR products were subjected to electrophoresis on a 3% NuSieve™ agarose gel (FMG Bioproducts, USA) or a 6% or 12% DNA-PAGE gel and stained with ethidium bromide (Fig. 1).

Table II. Overview of adverse effects observed in 115 patients with rheumatoid arthritis treated with MTX.

	n (%)	Withdrawals
Adverse effect	52 (45)	19 (17)
Elevation of transaminase[a]	20 (17)	2 (2)
Hair loss	11 (10)	1 (1)
Gastrointestinal intolerance	8 (7)	1 (1)
Skin rashes, itching	6 (5)	4 (3)
General fatigue	4 (3)	3 (3)
Pulmonary toxicity	5 (4)	5 (4)
Stomatitis	3 (3)	2 (2)
Leukopenia[a]	2 (2)	1 (1)
Lack of efficacy	1 (1)	1 (1)

Some patients experienced more than one adverse effects. [a]See text for definition.

Statistical analysis. Comparison of the clinical features between the two groups with or without MTX-related adverse effects was carried out using either χ^2 test or t-test. The relationship between the genotypes and clinical features was evaluated using χ^2 test. Serum levels of CRP before and after the MTX therapy were compared using paired t-test, and comparison with genotypes was analyzed using χ^2 test. A value of $P<0.05$ was considered statistically significant.

Results

Clinical features. MTX group consisting of 115 patients treated with MTX was divided into two subgroups according to the presence or absence of MTX-related toxicity. The demographic and clinical features of each group at study entry are described in Table I. In total, 52 of the 115 patients (45%) showed adverse effects during MTX therapy. Patients in both groups had been prescribed various doses (2-12 mg/week) of MTX, starting with the initial dose of 2-4 mg per week. If a satisfactory response to MTX could not be obtained judged by rheumatologists from clinical and laboratory findings, the MTX dosage was increased. In the group without adverse effects, 11 patients (17%) were receiving folic acid to prevent MTX toxicity in advance, while 21 patients (40%) with adverse effects received it mainly to minimize the adverse effects. There was no significant difference in the demographic and clinical features except for the folate supplementation ratio between the two groups.

Relationship between the genotypes and MTX toxicity. Of the 115 patients treated with MTX, 52 (45%) had one or more adverse effects. Among them, 19 (17%) discontinued MTX due to either a clinical adverse effect or a laboratory abnormality, and one patient due to lack of efficacy (Table II). Of the five patients with pulmonary toxicity, three suffered MTX-related pneumonitis, one had dry cough, and the remaining case suffered bacterial pneumonia after 6 months

Table III. Distribution of the *TYMS* genotypes in 167 patients with rheumatoid arthritis.
A, Tandem repeat polymorphism in the *TYMS* promoter region.

	Genotype			Frequency of 2R allele
	2R/2R	2R/3R	3R/3R	
MTX group (n=115[b])	4	34	75	0.18
With adverse effects (n=52[a])	2	15	34	0.18
Without adverse effects (n=63[a])	2	19	41	0.18
Non-MTX group (n=52)	1	9	42	0.11

2R/2R, homozygous for the double-repeat allele. 2R/3R, heterozygous with the double- and triple-repeats. 3R/3R, homozygous for the triple-repeat. [a]One patient each ([b]two of 115 MTX group patients) had a 3R/5R or a 3R/4R genotype, respectively.

B, Deletion polymorphism in the *TYMS* 3'UTR.

	Genotype			Frequency of 6-bp allele
	6 bp/ 6 bp	6 bp/ 0 bp	0 bp/ 0 bp	
MTX group (n=115)	5	52	58	0.27
With adverse effects (n=52)	2	25	25	0.28
Without adverse effects (n=63)	3	27	33	0.26
Non-MTX group (n=52)	3	21	28	0.26

6 bp/6 bp, homozygous for the wild-type allele. 6 bp/0 bp, heterozygous for the wild and 6-bp deletion allele. 0 bp/0 bp, homozygous for the 6-bp deletion allele.

from the commencement of the MTX therapy. Distribution of the *TYMS* and the *MTHFR* genotypes and allelotypes in the total 167 RA patients are summarized in Tables III-VI. The allele frequency of 2R was 0.18 in MTX group and that in the non-MTX group was 0.11 (P=0.0657). One patient each in the subgroups with or without adverse effects had a 3R/4R genotype for the *TYMS* polymorphism in the promoter region. The allele frequencies for the *TYMS* promoter, *TYMS* 3'UTR, *MTHFR C677T*, and *MTHFR A1298C* polymorphisms were similar between the MTX and non-MTX groups (Tables III and V). The relationship between the *TYMS* allelotypes and the adverse effects is shown in Table IV. No differences were seen in the allele frequencies of the both

Table IV. Association between *TYMS* polymorphisms and adverse effects of low-dose MTX in 115 patients (MTX group).

Polymorphism	With adverse effect n (%)	P-value
TYMS promoter region		
Without 2R allele (n=77)[a]	35 (45)	NS
With 2R allele (n=38)	17 (45)	
TYMS 3'UTR		
Without 6-bp allele (n=58)	25 (43)	NS
With 6-bp allele (n=57)	27 (47)	

2R, double repeat. 6 bp, the wild allele. [a]Two patients had a 3R/5R or a 3R/54 genotype, respectively. NS denotes not significant.

TYMS polymorphisms between the patients with or without adverse effects. Also for the *MTHFR C677T* and *A1298C* polymorphisms, there were no significant associations between the incidence of adverse effects and the allelotypes (Table VI). Distribution of the genotypes for each *TYMS* or *MTHFR* polymorphism was in Hardy-Weinberg's equilibrium.

Relationship between the genotypes and efficacy of MTX. Among the MTX-group, the efficacy of MTX was evaluated in 93 patients who could be assessed on the serum CRP levels before and more than two months after the commencement of MTX therapy. Patients who discontinued MTX due to the adverse effects within two months were excluded. The CRP levels after the MTX therapy (mean ± SD: 1.2±1.2 mg/dl) were significantly lower than those (3.0±2.2 mg/dl) before the therapy (P<0.0001), indicating that the dose of MTX maintained in each case was generally sufficient.

The 93 patients were divided into two subgroups according to the MTX dosage required, above or below 6 mg/ week. Between the two groups, there was no significant difference in the demographic or disease variables, concomitant drugs such as NSAIDs, corticosteroid, or other DMARDs, or the dosage of corticosteroid, except for the percentage of RF-positive patients (36 vs 82%, respectively). Statistical analysis on the association between the genotypes and the required MTX dosage showed that the patients homozygous for the 3R allele (without 2R) of the *TYMS* gene required significantly higher doses of MTX than the others (with 2R) (P=0.033, Table VIIA). On the other hand, there was no significant difference concerning the *TYMS* 3'UTR polymorphism and the *MTHFR C677T* and *A1298C* polymorphisms (Table VII).

Among the 93 patients, the association between the genotypes and the improvement in the serum CRP levels after the MTX therapy was assessed in 64 patients who showed CRP ≥1.0 mg/dl before the MTX therapy. The incidence of ≥50% improvement was significantly higher in

Table V. Distribution of the *MTHFR* genotypes in 167 patients with rheumatoid arthritis.
A, *MTHFR C677T* polymorphism.

	Genotype			Frequency of *677T* allele
	CC	CT	TT	
MTX group (n=115)	46	47	22	0.40
With adverse effects (n=52)	24	18	10	0.37
Without adverse effects (n=63)	22	29	12	0.42
Non-MTX group (n=52)	19	21	12	0.43

CC, homozygous wild; CT, heterozygous; TT, homozygous variant at position 677 in the methylenetetrahydrofolate reductase gene.

B, *MTHFR A1298C* polymorphism.

	Genotype			Frequency of *1298C* allele
	AA	AC	CC	
MTX group (n=115)	80	29	6	0.18
With adverse effects (n=52)	36	14	2	0.17
Without adverse effects (n=63)	44	15	4	0.18
Non-MTX group (n=52)	37	14	1	0.15

AA, homozygous wild; AC, heterozygous; CC, homozygous variant at position 1298 in the methylenetetrahydrofolate reductase gene.

Table VI. Association between *MTHFR* polymorphisms and adverse effects of low-dose MTX in 115 patients with rheumatoid arthritis (MTX group).

Polymorphism	With adverse effects n (%)	P-value
C677T (n)		
Without *677T* (46)	24 (52)	NS
With *677T* (69)	28 (41)	
A1298C (n)		
Without *1298C* (80)	36 (45)	NS
With *1298C* (35)	16 (46)	

NS, not significant.

Table VII. Association between the polymorphism and the MTX dosage in 93 patients treated with MTX for more than 2 months.
A, *TYMS* polymorphism.

Dose of MTX	Promoter region[a]		3'UTR	
	Without 2R	With 2R	Without 6 bp	With 6 bp
<6 mg/week (n=34)	18	16	14	20
≥6 mg/week (n=59)	44	15	30	29

2R, double repeat. 6 bp, the wild allele. [a]P=0.033.

B, MTHFR polymorphism.

Dose of MTX	*C677T*		*A1298C*	
	Without *677T*	With *677T*	Without *1298C*	With *1298C*
<6 mg/week (n=34)	11	23	26	8
≥6 mg/week (n=59)	21	38	41	18

The *677T* and the *1298C* are variant alleles at position 677 or 1298 in the methylenetetrahydrofolate reductase (*MTHFR*) gene, respectively.

patients homozygous for the deletion allele (0 bp/0 bp, Table VIII). Then, the combination of the 3R/3R genotype for the *TYMS* promoter region, which was associated with higher maintenance dose of MTX, and the genotypes with the 6-bp insertion allele (6 bp/6 bp and 6 bp/0 bp) for the 3'UTR, that showed lower response in CRP level, was compared to others. The RA patients with this combination demonstrated a significantly lower incidence of ≥50% improvement in CRP levels (54.2 vs 79.6%, P=0.0242).

Haplotype. The association between the polymorphisms within the *TYMS* gene or *MTHFR* gene was analyzed in the total 167 individuals with RA. The genotype homozygous for the 3R allele (3R/3R) was significantly associated with the genotype homozygous for the deletion allele (0 bp/0 bp) in the *TYMS* 3'UTR (P=0.0003, Table IX). Concerning the *MTHFR* polymorphisms, there was no linkage disequilibrium (data not shown).

Ethnic differences in the distribution of the genotypes. Among the 167 Japanese patients with RA, the frequency of the wild allele (2R) for the polymorphism in the *TYMS* promoter region was 0.16, while it was reportedly 0.40 in Caucasians (12) (P<0.0001, Table X). The frequency of the vaiant allele (0 bp) for the *TYMS* 3'UTR polymorphism was 0.73 in the present Japanese individuals, while it was 0.29 in the Caucasians (P<0.0001) (4). Also concerning the *MTHFR*

Table VIII. Association between the *TYMS* genotypes and % improvement of serum CRP levels in 64 patients who showed CRP ≥1.0 mg/dl before the MTX therapy.

	Promoter region			3'UTR[a]		
% Change of CRP[b]	2R/2R	2R/3R	3R/3R	6 bp/6 bp	6 bp/0 bp	0 bp/0 bp
<50% (n=19)	0	4	15	2	13	4
≥50% (n=45)	1	17	27	2	21	22

[a]P=0.0383, 0 bp/0 bp genotype compared with other genotypes. [b]% Change of CRP: 100 x (CRP after therapy - CRP before therapy)/CRP before therapy.

Table IX. Association between the polymorphism in the promoter region and that in the 3'UTR of the *TYMS* gene.

	Promoter region	
3'UTR	Homozygous variant (3R/3R)	Others
Homozygous variant (0 bp/0 bp)	71	15
Others	46	35

P=0.0003.

polymorphisms, there were ethnic differences as previoiusly reported (13) (Table X).

Discussion

In the present study, we found that the RA patients homozygous for the triple repeat (3R/3R) allele of the polymorphism in the *TYMS* promoter region required higher dosage of MTX to control their disease activity than those with other genotypes (2R/2R and 2R/3R). Weekly MTX therapy for RA was demonstrated to show a dose-response relationship for efficacy (14), and the effective dosage of MTX varies among the individual case. The present finding suggests that the *TYMS* promoter genotypes may be related to the efficacy of

Table X. Ethnic differences in the distribution of the genotypes and alleles of the polymorphisms in the *TYMS* and *MTHFR* genes.

Ethnic group	n	Genotype			Allelotype	
TYMS promoter		2R/2R	2R/3R	3R/3R	2R	3R
Japanese	167	3%	26%	70%	0.16	0.84[b]
Caucasian	96	19%	43%	38%	0.40	0.60
TYMS 3'UTR		6 bp/6 bp	6 bp/0 bp	0 bp/0 bp	6 bp	0 bp
Japanese	167	5%	44%	51%	0.27	0.73[b]
Caucasian	95	48%	44%	7%	0.71	0.29
MTHFR C677T		CC	CT	TT	*677T*	
Japanese	167	39%	41%	20%	0.41[a]	
Caucasian	159	46%	43%	11%	0.33	
MTHFR A1298C		AA	AC	CC	*1298C*	
Japanese	167	70%	26%	4%	0.17[b]	
Caucasian	159	44%	47%	9%	0.32	

Japanese, patients with rheumatoid arthritis in the present study. Caucasian, individuals in general population or those with various diseases (4,12,13). [a]P=0.0339; [b]P<0.0001.

low-dose MTX in RA patients. A reporter gene linked to the *TYMS* promoter sequence with the triple repeat showed 2.6-fold higher expression activity than that with the double repeat in an *in vitro* expression study (3). The *TYMS* mRNA in tumors homozygous for the triple-repeat sequence is reportedly more efficiently translated than that with the double-repeat sequence (15). TS is also a primary target for cancer chemotherapeutic drugs, such as 5-fluorouracil (5-FU). Recent studies suggested that the tandem-repeat polymorphism in the *TYMS* promoter region is one of the determinants for response and toxicity of 5-FU based chemotherapy (16). More recently, Krajinovic *et al* reported the relationship between this polymorphism and outcome of acute lymphoblastic leukemia children receiving high-dose MTX (4 g/m^2) (17): individuals with the 3R/3R genotype had a poorer outcome than those with other genotypes. This study indicates that the effect of high-dose MTX, which is partly derived from its inhibitory effect on the TS activity, is closely correlated with the genotypes of the *TYMS* tandem-repeat polymorphism as well as the 5-FU. Our results indicate that also in the low-dose MTX therapy, its effects partly depend on the genotypes of the *TYMS* tandem-repeat polymorphism.

Moreover, we found that the RA patients homozygous for the variant deletion allele of the *TYMS* 3'UTR polymorphism showed significantly higher CRP response to MTX therapy. This allelotype was recently reported to be associated with lower *TYMS* mRNA expression in tumor cells (Lenz *et al*, Proc Am Assoc Cancer Res 43: abs. 660, 2002). Since individuals with lower TS expression might be more sensitive to the MTX, our result is compatible with this evidence.

On the contrary, no association was observed between the MTX toxicity and any of the *TYMS* or *MTHFR* polymorphisms examined. Currently, it was speculated that some toxicity of MTX, especially such as hepatotoxicity, is possibly mediated by homocysteine metabolism (1). In fact, it was reported that patients undergoing low-dose MTX therapy showed higher plasma homocysteine levels (18), and that the 3R/3R genotype for the polymorphism in the *TYMS* promoter region was associated with elevated plasma homocysteine levels among individuals with low dietary folate intake (19). Then, one would expect that patients homozygous for the *TYMS* triple-repeat allele (3R/3R) or having 6-bp insertion allele (6 bp/6 bp or 6 bp/0 bp), which were reported to show higher TS expression, might have less toxicities than those with others, especially in tissues with a high cell turn over (bone marrow and gastrointestinal tract). Therefore, we examined the association between the *TYMS* genotypes and adverse effects of hair loss, gastrointestinal intolerance, stomatitis, and leukopenia. But we could not observe significant difference between any *TYMS* polymorphism and toxicities. Some explanations for this discrepancy can be considered: first, 17% of the patients treated with MTX without any adverse effects had been prescribed folic acid to prevent MTX-related toxicity in advance. The effect of folic acid supplementation has been shown to be very useful in lessening toxicity during low-dose MTX therapy (20). Second, additional DMARDs had been concurrently prescribed for >30% of the patients. These additional DMARDs might have influenced the development of MTX-related toxicity. However, since the mechanism of developing toxicity is heterogeneous, further studies with larger sample size might be necessary to draw the conclusion on the relationship between the genotypes and adverse effects.

It has been reported that the *C677T* and *A1298C* polymorphisms in the *MTHFR* gene were associated with the efficacy or the toxicity of MTX (21,22). However, we did not find any associations between them. We may speculate that the effects of *TYMS* polymorphisms on the sensitivity to the low-dose MTX therapy may be stronger than that of *MTHFR* polymorphisms.

We found significant differences in the distribution of the *TYMS* 3'UTR genotypes between the Japanese individuals with RA in the present study and Caucasians reported in general populations (4) (Table X). The frequency of the deletion allele (0 bp) was 73% in Japanese RA patients and 29% in Caucasians in general population, indicating that there is an apparent ethnic variation in the distribution of the genotypes for the 3'UTR polymorphism as well as that in the promoter region (12) of the *TYMS* gene or those in the *MTHFR* gene (13).

In conclusion, the wild allele for the polymorphism in the promoter region (2R) and the variant allele for the 3'UTR (0 bp) seem to be the significant predictors of the high sensitivity to low-dose MTX therapy. Lack of association between these polymorphisms and MTX-related adverse effects indicates that the mechanism in the development of toxicity may be different from that in the expression of efficacy with the low-dose MTX therapy. In addition, existence of apparent ethnic variations in the distribution of their genotypes may indicate the importance of genotyping to predict and determine the appropriate dose in the low-dose MTX therapy, and our results may provide useful information for the individualized therapy in patients with RA.

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