▷免疫検査の最近の話題(2)◁

関節リウマチ(RA)

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Topics of Immunological Tests for Rheumatoid Arthritis

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To prevent joint destruction, it is important to diagnose RA early and to speculate the prognosis. Several new laboratory tests have been developed for this purpose. In addition to rheumatoid factor (RF), IgG-RF, anti-agalactosyl IgG antibodies (CARF), and matrix metalloproteinase 3 (MMP-3) have become available as diagnostic tests for RA. Among them, anti-cyclic citrullinated peptide antibodies (anti-CCP antibodies) have the sensitivity and specificity of 81.0% and 92.4%, respectively, which are superior to other laboratory tests by ROC analysis. Moreover, the specificity of anti-CCP antibodies to diagnose early RA was much higher than that of RF, although the sensitivity of CARF was slightly high compared with that of RF.

In contrast, MMP-3 is thought to be an evaluative test for the activity of RA because a significant correlation was found between MMP-3 and CRP, but MMP-3 has a possibility as a prognostic test to know the joint damage of RA. We have shown that the progression of joint damage (Sharp score) was faster in MMP-3-positive patients than negative patients. Anti-CCP antibodies was also reported to associate with the progression of joint damage and may be used also as a prognostic test.

We next examined how efficiently was the diagnosis of RA made by combining these laboratory tests. Although anti-CCP antibodies are highly specific to RA, the specificity of RF was not so high but became up to 92% when combined with MMP-3. In order to diagnose RA efficiently, we may firstly examine RF, next MMP-3 if RF is positive, and anti-CCP antibodies if RF is negative.

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【Key Words】 rheumatoid arthritis(関節リウマチ), rheumatoid factor(リウマトイド因子), anti-cyclic citrullinated peptide antibodies(抗 CCP 抗体), matrix metalloproteinase-3(マトリックスメタロプロテイナーゼ-3), diagnostic tests(検査診断)

関節リウマチ(RA)は，関節の炎症と滑膜増殖による骨軟骨の破壊を特徴とする原因不明の慢性炎症性疾患である。RA 患者は，持続性の痛み，関節機能障害，生命予後不良という三つの大きい問題を有している。近年メトトレキサートの普及や抗 TNFα 薬などの生物製剤が開発され，RA 治療の考え方は「関節機能を長く保つ」から「関節障害を起こさない」へと，大きい変遷を遂げつつある。関節機能障害を

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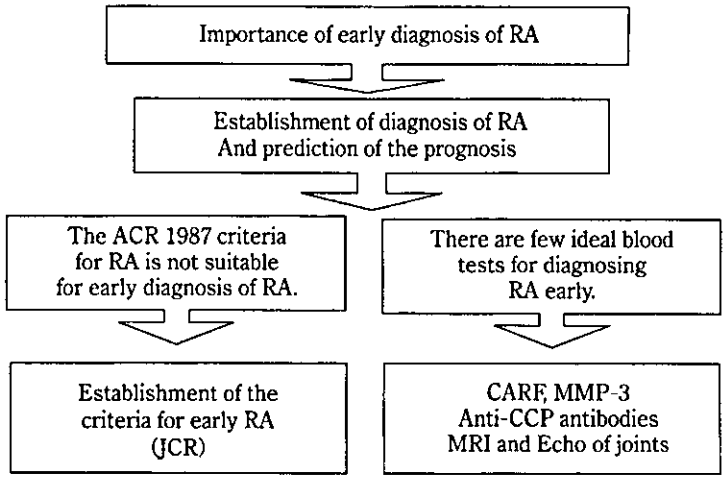


Figure 1 Importance of early diagnosis of RA and its strategy.

防ぐには，発症早期からのDMARDの使用や難治例には多剤併用療法や生物製剤を適切に使用することが重要である。これらの事実を踏まえて2002年にアメリカリウマチ学会(ACR)によるRA治療のためのガイドラインがアップデートされたが，新しい治療の考え方とともに，早期診断，活動性の把握，予後の推定の重要性が強調されている[1]。

RAを早期に確実に診断するためには，優れた診断基準と臨床検査が必須である(**Fig. 1**)。RAの診断はACRの1987年分類基準に拠ることが多いが，この分類基準は感度特異度とも90%以上と優れたものであるが，早期診断には不向きであることが指摘されている[2][3]。我が国ではリウマチ学会や厚生省研究班が中心となり，RA早期診断のための基準が提案されているが，その感度や特異度も十分とは言えない[4]。RA診断における検査の意義と有用性は，早期診断，活動性や予後の推定，治療効果や副作用の判定などである。RAの血清診断においてリウマトイド因子(RF)の感度は約80%とまずまずであるが，結核やウイルス性肝炎などの慢性感染症患者にもかなりの陽性者が存在することが指摘されている[5]。このようなことから，感度・特異度がともに優れ早期のRAを確実に診断できる検査(diagnostic tests)，活動性や関節炎などの治療効果の判定のための検査(evaluative tests)，およびRAの進行性や関節の予後予測ができる検査(prognostic tests)の開発が望まれてきた。

Table 1に示すように従来diagnostic testsとしてはRF(IgMクラス)が用いられてきたが，IgGクラスのRF(IgG-RF)や抗ガラクトース欠損IgG抗体(CA・RF)も開発使用されている[6]。evaluative testsとしては，赤沈やCRPなどの炎症マーカーが用いられてきたが，これらの炎症検査は必ずしも関節の機能的予後を反映していないことから，MMP-3が注目されている[7]。さらに，RAに特異性が高いdiagnostic testとして，抗環状シトルリン化ペプチド抗体(抗CCP抗体)が新しく開発された。本稿ではこれらの検査とその使い方について，我々の検討を含めて紹介する。

I. リウマトイド因子(RF)

従来のRFはIgMクラスであり，上記のACRのRA分類基準や日本リウマチ学会の早期RA診断基準にも含まれている[4]。このIgMクラスRFはRAのみならず，高齢者や慢性炎症患者，特にRA以外のリウマチ性疾患でも高率に検出されることから，RAの診断に混乱をもたらした一因でもある[5]。IgGクラスRF(IgG-RF)は，特に高値の場合はRAの可能性が高いと考えられ，活動性と相関することから，治療効果のevaluative testとしても用いられる[8]。

IgGのCH2ドメインにはN-グリコシド型糖鎖が結合しているが，糖鎖構造解析により，RA患者由来のIgGはこの糖鎖のうちガラクトースを欠いた糖鎖の割合が健常人に比し有意に高く，その増加が予後の指標となることが報告された。さらにRA患者血中のRFはガラクトース欠損IgGに対する親和性が高いことから，RAの発症やRFの産生にこのガラクトース欠損IgGが自己抗原として関与している可能性が推察され，これを抗原としたRF測定法抗ガラクトース欠損IgG抗体(CA・RF)が開発された。

Table 1 Laboratory tests for rheumatoid arthritis

Diagnostic tests	Blood tests	RF, IgG-RF, Anti-agalactosyl IgG antibodies Matrix metalloproteinase-3 (MMP-3) Anti-CCP antibodies, Anti-calpastatin antibodies
	Other tests	Plain roentgenography , Scintigraphy MRI, Ultrasonography Synovial fluid analysis, Arthroscopy
Evaluative tests	Blood tests	ESR, CRP, Serum amyloid-A (SAA) IgG-RF, RF, MMP-3 Cartilage oligomeric matrix protein (COMP)
	Other tests	Plain roentgenography
Prognostic tests	Blood tests	ESR, CRP HLA-DR4, PADI4 polymorphism RF, Anti-CCP antibodies, MMP-3
	Other tests	Plain roentgenography

Table 2 Positivity of diagnostic tests for RA (%)

	RF	CARF	IgG-RF	Anti-CCP Abs
RA (n=79)	69.6	75.9	27.8	81.0
Healthy subjects (n=202)	3.5	6.4	2.0	3.0
Non-RA † (n=105)	22.9	31.4	11.4	7.6
Chronic patients ‡ (n=124)	22.6	—	—	0.8

† non-RA: SLE, SjS, PSS, PM/DM, MCTD.
‡ Patients with chronic inflammation such as malignancy and viral hepatitis.

RA での陽性率が高く，特に早期 RA の診断にも有用であるとされた[9]。

Table 2 に示すように，RF は，RA 患者での陽性率 69.6%と高率であるが，RA 以外のリウマチ性疾患患者，特にシェーグレン症候群では高値を示す例が多く見られ，約半数が陽性になる。また悪性腫瘍やウイルス性肝炎などの慢性炎症性疾患患者における陽性率も 22.6%と高かった。IgG-RF(エイテスト IgG-RF，三光純薬)では RF とは異なって非 RA での陽性例は少ないが，RA での陽性率は 27.8%と低率であった。一方，CA·RF(ピコルミ CA·RF，三光純薬)は RA 患者での陽性率は 75.9%と RF より若干高かったが，シェーグレン症候群での陽性率は 63%と非 RA のグループでの陽性例が多く認められた[6]。

次に早期 RA 患者での陽性率を比較するために，RA 患者を発症 2 年未満と 2 年以上の二群に分けて各検査の陽性率を比較した(**Table 3**)。従来から言われているように早期 RA での RF 陽性率は 56.3%と低かったのに対して，CA·RF は発症 2 年未満の患者においても 75.0%が陽性であり，RA 患者の早期診断での役割が期待される。一方 IgG-RF は発症 2 年未満の陽性率が 43.8%であるのに対して，2 年以上の陽性率は 23.8%と低かった。IgG-RF は活動性と相関する事が知られており，2 年以上の患者にはコントロール良好の患者が多かったためと考えられる。CA·RF は IgG や IgA クラスの RF も検出することから，IgM 以外の RF の検出が早期 RA の診断に有用である可能性も考えられる。

Table 3 Comparison of the positivity for various RA tests between patients with early and advanced RA (%)

	<2 years after the onset (n=16)	≥2 years after the onset (n=63)
RF	56.3	73.0
CA·RF	75.0	76.2
IgG-RF	43.8	23.8
Anti-CCP Abs	68.8	84.1
MMP-3	75.0	68.3

II. 抗 CCP 抗体

RA に特異的な自己抗体として，以前より抗ケラチン抗体や抗核周囲因子抗体の存在が報告されていた[10][11]。その後，これらの対応抗原が上皮組織のフィラグリンであることが明らかにされた。フィラグリンは上皮の角質化に伴って，その分子を構成するアルギニンが脱イミノ修飾を受けシトルリンに変わりシトルリン化フィラグリンとなるが，RA 患者血清はこのシトルリン化フィラグリン分子に対して特異的に反応することが報告された[12]。2000 年 Schellekens らは，フィラグリン配列からシトルリンを含んだ環状ペプチドを合成し，これを抗原とした抗体(抗シトルリン化環状ペプチド抗体；抗 CCP 抗体)が RA 患者に特異的であることを報告した[13]。

Visser らは 524 例の発症早期の多発関節炎患者を 2 年間経過観察し，関節破壊を来たす症例(RA)を早期に鑑別するためには，抗 CCP 抗体を取り入れた診断基準モデルの方が，従来の ACR の分類基準に比べて感度・特異度の点で優れていたことを明らかにした[14]。さらに 2 週間以内に診断がつかなかった早期関節炎患者 318 名を 3 年間前向きに follow したところ，抗 CCP 抗体陰性者 249 名中 63 例(25%)が RA と診断されたのに対し，陽性者 69 例では実に 64 例(93%)が RA と診断された[15]。実際，RA 発症者においてはその数年前から抗 CCP 抗体や RF が陽性である事が，血液バンクに保存された血液の解析から明らかにされている[16][17]。RA 発症の予測因子として報告されている HLA-DR4 や PADI4(peptidyl-arginine deiminase 4)多型などの遺伝的因子，あるいは血清学的因子である RF の odds ratio が 2 程度であるのに対して，抗 CCP 抗体の odds ratio は 30 程度と非常に高い[15][18]。

我々も ELISA 法(DIASTAT Anti-CCP，MBL 社)を用いた検討を行い，抗 CCP 抗体の抗体価分布を調べた(**Fig. 2**)。RA 患者では高濃度に分布し，5 U/ml をカットオフ値とした場合の RA 患者での陽性率は 76.0%と高率であった。健常者や変形関節症患者での陽性率は 5%以下であり，非 RA 患者での陽性率も 7.6%と低く，慢性炎症性疾患でもほとんど陽性とはならなかった(**Table 2**)。早期 RA での陽性率は，RF よりは高いが CA・RF よりは低い結果であった(**Table 3**)[6]。しかしながら，RF 陰性の RA 患者 23 例のうち，8 例(35%)で抗 CCP 抗体が陽性であり，RF との併用による早期診断での有用性などが期待される。抗 CCP 抗体は，本邦では保健適用されていないため，実際の臨床の場ではまだ使用することはできないが，RF と比べて感度は同等以上で特異度は明らかに高く，RA の早期鑑別診断における有用性は明らかである[19][20]。

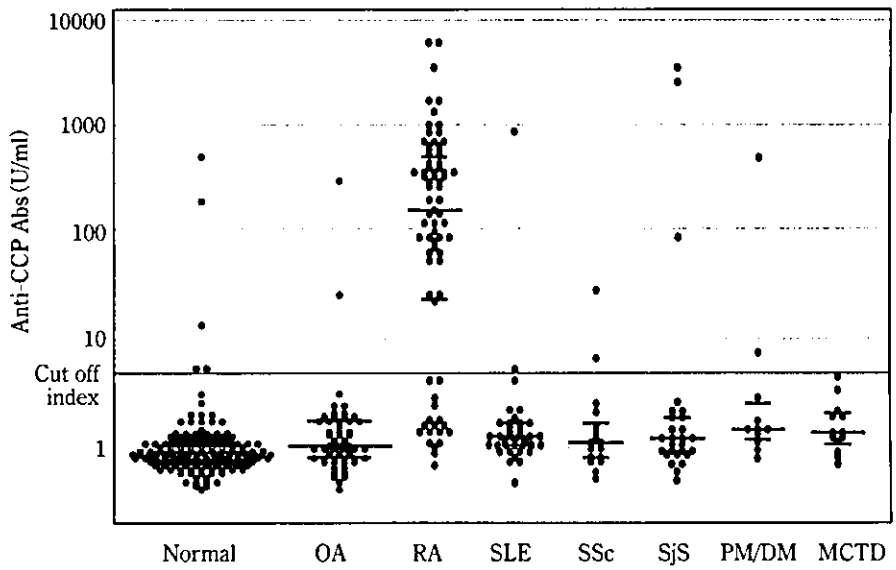
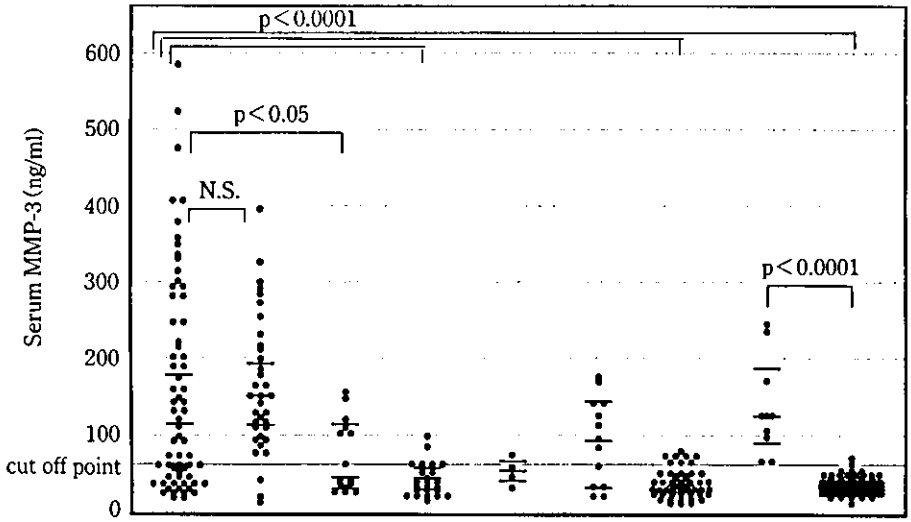


Figure 2 Levels of anti-CCP Abs in various rheumatic diseases.
Horizontal bars indicate the respective median values and the 25- and 75-percentile values.
OA: Osteoarthritis.



	RA	SLE	PSS	SjS	PM/DM	MCTD	OA	Kidney disease	Normal control
Positivity (%)	64.0	86.8	47.1	22.2	54.5	61.5	16.4	93.8	5.8

Figure 3 Levels of serum MMP-3 in various rheumatic diseases.
Only women were examined because the levels were significantly higher in men than women.

III. マトリックスメタロプロテイナーゼ-3 (MMP-3)

MMP-3 は IL-1 などの刺激により，滑膜細胞や軟骨細胞あるいはマクロファージなどで産生誘導される蛋白分解酵素で，細胞外マトリックス，特にプロテオグリカンを分解し軟骨破壊を起こすとともに，I 型や II 型コラーゲンを分解する MMP-1 の活性化を誘導するなど関節破壊に深く関与している。RA 患者関節液には大量の MMP-3 が含まれていることが報告された[21]。1995 年小幡らにより MMP-3 の EIA 法が開発され，RA 患者の血清中にも MMP-3 が増加し，活動性の指標となること，RA の早期診断における有用性が呈示された[22][23]。

我々は ELISA 法(パナクリア MMP-3「プレート」，第一化学薬品)により血清 MMP-3 濃度を測定した。健常人男性 63 例の血清 MMP-3 濃度は 88.0±28.6ng/ml(平均±SD)と，女性の 34.0±10.1ng/ml(92 例)に比べ有意に高値であった(p<0.0001)。我々が行った各種膠原病と腎疾患患者などでの MMP-3 濃度分布(性差があるため，女性のみの分布)を **Fig. 3** に示した[6]。**Fig. 3** に示すように，RA 患者では高値を示す例が多く OA やシェーグレン症候群に比べて有意に高値であった。しかしながら他の膠原病特に SLE で高値に分布し，陽性率は RA より SLE の方が高かった。この点に関して，未治療の SLE 患者では血清 MMP-3 は高くなく，ステロイド投与が血清 MMP-3 を増加させるとの報告もあり，我々の症例も多くはステロイド服用中であり今後の検討を要する[24]。また，腎疾患患者でも多くの例で高値を示すことから，RA の診断や活動性の評価に用いる場合は注意が必要である。

Table 3 に示したように MMP-3 の陽性率は，発症 2 年未満の早期 RA で 75.0%と，早期診断における有用性も期待される。発症 2 年以上の患者の陽性率が 68.3%と低いのは，治療により陰性化した例も含まれているためと考えられる。

IV. RA の Evaluative tests と Prognostic tests

RA の疾患活動性を評価するための血液検査としては，赤沈や CRP が用いられている。同じ急性期反応性蛋白である血清アミロイド A も活動性に応じて増減するが，その濃度と合併症であるアミロイドーシス発症との直接的な関係は証明されていない[25]。IgG-RF は活動期に高いことから病勢との関連が示唆されるが，陽性率があまり高くなく，治療に反応してすぐに陰性化する例が多い。

RA 患者において，血清 MMP-3 値は CRP と有意の正相関(n=75，r=0.556，p<0.0001)を示し，経

時変化でも両者の動きがほぼ同期する例が多い事から，MMP-3は活動性の指標となると考えられる。しかしながら経過を追って観察すると，CRPが陰性化した後もMMP-3は高値を続ける例も散見される。我々は抗TNFα(Infliximab)療法を行った9例について継時的にCRPやMMP-3を測定したところ，投与開始14週後では両者とも投与前と比べて有意に低下していた。しかしながら，CRPがほぼ陰性化してもMMP-3が150 U/ml以上の高値を続ける症例が3例あった。関節変形を阻止するためにはMMP-3の正常化が必要かどうかなど，今後の検討が待たれる。

2000年山中らは，早期RA患者の6ヵ月後や12ヵ月後の関節X線所見(Larsen grade)が進行した例ほど，初回測定時のMMP-3濃度が高いことから，初期の血中MMP-3濃度が関節病変の予後予測に有用であると報告している[26]。我々のRA患者の検討でも，Steinblockerの病期Stage分類がIからIVに上がるに従って，それぞれ28.6%，50.0%，66.7%，84.6%とMMP-3の陽性率も上昇した($p<0.01$)[6]。またRA症例18例について経時的に手の関節X線を観察すると，初回撮影時にMMP-3が基準範囲より高値であった6例では基準範囲内であった12例に比べて関節病変の進行(Sharp score)が有意に早かった。これらのことから，MMP-3は活動性のみならず，関節病変の予後予測に使えるマーカーとして期待できる。

関節予後不良のマーカーとしては従来から，RF高値，CRPや赤沈の亢進，HLA-DR4などのshared epitope(SE)，関節X線での変化の存在などが知られている。IgG-RFやCA・RFなどについて，RF以上の意味があるかどうかは不明である。抗CCP抗体については前述のように，RAを中心としたerosive arthritisをnon-erosive arthritisから鑑別する意義は疑いのないところであるが，RAの中で関節の予後不良の指標となるかどうかについては明確ではない。最近van Gaalenらは，抗CCP抗体と上記のHLAのSEを有する患者は，どちらかを有する患者や両者を有しない患者に比べて関節破壊の進行が急速であることを示した[27]。さらに多変量解析で抗CCP抗体はIgM-RFとは独立した因子であった。

また最近，早期RA患者について，抗CCP抗体がX線上の関節障害と相関することが報告されている[28]。我々の継時的な検討でも，抗CCP抗体陽性16例のRA患者では手の関節病変(Sharp score)が陰性患者4例に比べて有意に早く進行していた($p<0.05$)。これらのことから抗CCP抗体は関節予後推定の指標となると考えられる。

V. 検査の使い方と意義

いくつかのRAの診断のための新しい優れた検査法が開発され，RAの血清診断や血液検査による予後推定が可能となりつつある。これらの検査をどのように組み合わせて使うのが良いのかは大変重要であり，臨床疫学的なエビデンスに基づいたガイドラインの作成が望まれる[3)29)]。現在我々もいくつかの施設との共同研究で，臨床的エビデンスの収集と対費用効果を考えた検査診断の検証によるガイドラインの作成を目指している。

RF，CA・RF，IgG-RF，抗CCP抗体，MMP-3の5種類の検査を様々に組み合わせて，RA患者の診断のための効率を検討した[6]。RF，CA・RF，IgG-RFの三つはオーバーラップが多かった。RA 75例中49例はRF，CA・RFおよび抗CCP抗体のいずれもが陽性であり，RF(+)抗CCP抗体(−)は3例しか認めなかったのに対して抗CCP抗体(+) RF(−)は8例存在した。一方，RF，抗CCP抗体，MMP-3の3者が陽性の患者は75例中36例と少なく，抗CCP抗体(−)でMMP-3(+)の患者は8例，RF(−)でMMP-3(+)の患者は12例存在した。

このようなことからRFや抗CCP抗体などの抗体系の検査にMMP-3を加えることにより，診断の効率が上昇すると考えた。**Fig. 4**に示すように，抗CCP抗体は単独でも特異度は高い検査であるが，RFはMMP-3と併用することにより両者陽性の場合特異度が92%と高くなることがわかった。RA診断のためにはまずRF検査を行い，陽性ならMMP-3を検査し，陰性なら抗CCP抗体を検査することにより，効率よく血清診断ができると考えている。現時点では抗CCP抗体検査は保険収載されておらず，大規模な前向き研究による今後の検証が必要である。また抗CCP抗体が認可されたとしても，血液検査だけでRAを診断することは不可能であり，診断は臨床所見とともに，画像診断など他の検査を総合的に判断して行う必要があることは言うまでもない。

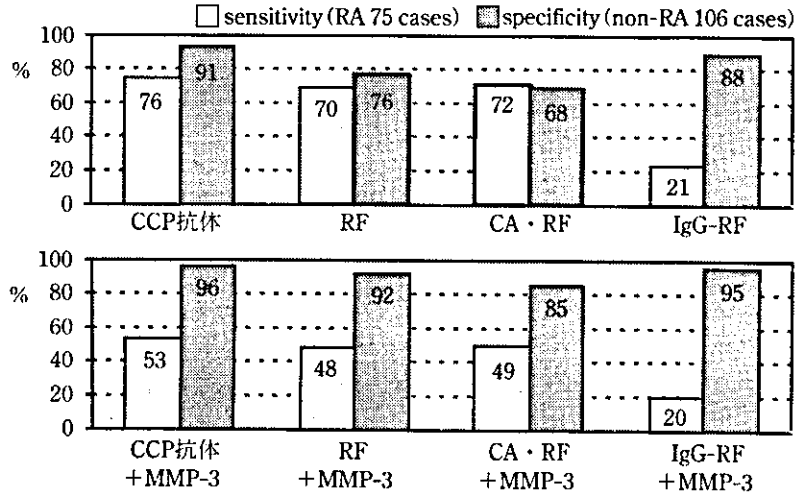


Figure 4 Sensitivity and specificity of combinatory tests.
Sensitivity and specificity of anti-CCP Abs, RF, CARF, and IgG-RF for RA and non-RA were compared when MMP-3 was combined. Numbers in the bars indicate %.

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RCM

Effect of mutated transporters associated with antigen-processing 2 on characteristic major histocompatibility complex binding peptides: analysis using electrospray ionization tandem mass spectrometry

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A novel allele of transporters associated with the antigen-processing (TAP) 2 gene, *TAP2*Bky2* (Val^{577}), is significantly increased in Japanese patients with Sjögren's syndrome (SS), and has a strong association with SS-A/Ro autoantibody production in SS and autoantibody including anti-SS-A/Ro and anti-U1 RNP antibody in systemic lupus erythematosus (SLE). To determine the influence of this natural mutated TAP on peptides loaded onto MHC class I, we analyzed the repertoire of peptides loaded onto MHC class I on transfectants with TAP1 and TAP2 or mutated TAP2 by electrospray ionization tandem mass spectrometry (ESI-MS/MS). After comparison of the peptide profiles we identified three peptides from only mutated TAP transfectants. Moreover, one of these peptides is derived from snRNP A, which is a target for anti-U1 RNP antibody. To our knowledge this is the first report to show that the natural mutation of TAP2 changes the peptide profile loaded onto MHC class I molecules. Copyright © 2004 John Wiley & Sons, Ltd.

Major histocompatibility complex (MHC) class I molecules present intracellular peptides to cytotoxic CD8-positive T cells. Many of these peptides are generated by proteasome from newly synthesized cellular or viral proteins. Peptides are subsequently transported into the endoplasmic reticulum (ER), where they bind to MHC class I molecules. The peptide translocation across the ER membrane is mediated by the specialized ABC (ATP-binding cassette) transporters associated with antigen processing (TAP). The TAP molecule is composed of two subunits, TAP1 and TAP2, which form a functional heterodimer.[1–3] TAP1 or TAP2 transfectants using mammalian cells, insect cells, or yeasts, suggest that neither TAP1 nor TAP2 can form a functional homodimer, indicating that only the heterodimer is functional.[4,5] TAP contains three structural domains, a heterodimeric pore domain, followed by a heterodimeric domain and two hydrophilic nucleotide-binding domains (NBD). TAP1 and TAP2 showed the highest homology in a stretch of 200 amino acids located at the C-terminus.

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The best characterized human ABC transporter is the cystic fibrosis transmembrane regulator (CFTR) that functions as an ion (chloride) channel. The natural point mutations of the CFTR gene cause cystic fibrosis by closing the chloride channel permanently[6] or lowering the chloride flow rate through the channel.[7] TAP, another ABC transporter, has been regarded as the crucial molecule in selecting the peptides presented by MHC class I molecules and in corresponding with the immune system. Therefore, the genetic polymorphism of TAP genes has been studied in several autoimmune diseases in order to assess their contribution to the risk of disease.

Previously, we found a novel mutation at codon 577 (ATG to GTG: Met to Val) in exon 9 of the TAP2 gene.[8] Based on this mutation a new *TAP2*Bky2* allele was identified. We found a significantly increased frequency of *TAP2*Bky2* allele in Japanese patients with Sjögren's syndrome (SS) and a strong association with SS-A/Ro antibody production in Japanese SS patients.[8] In Japanese patients with systemic lupus erythematosus (SLE), *TAP2*Bky2* is associated not only with the anti-SS-A/Ro antibody, but also anti-SS-B/La, anti-U1 RNP and anti-Sm antibody production.[9]

The close association of specific MHC class I subclasses with autoimmune diseases has been reported. This may be explained by the fact that each MHC class I molecule has an allele-specific motif and characteristic residues. Therefore, the peptides loaded on MHC class I vary with MHC class I

subclass, constituting unique epitopes for CD8-positive T cell recognition. One can easily imagine that peptides presented by MHC class I are as important as the subclass of MHC molecules in antigen presentation.

Advances in technology in the field of tandem mass spectrometry enable us to directly identify peptides bound to MHC molecules. In *in vitro* analysis, artificially introduced point mutations of the TAP1 or TAP2 subunit affect the function of peptide transporters.[10,11] However, there has been no analysis of whether the natural mutation of the TAP2 gene might affect the repertoire of peptides loaded onto MHC class I molecules. In this study we investigated the influence of this natural TAP2 mutation to the repertoire of peptides loaded onto MHC class I on transfectants with TAP1 and TAP2 or *TAP2*Bky2*, using mass spectrometric techniques.

EXPERIMENTAL

Construction of TAP1 and TAP2 mutants

The vector pREP8Δ containing human TAP2 cDNA was kindly provided by Dr. Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Targeted mutagenesis to generate the desired mutation at codon 577 (ATG to GTG) was performed on single-stranded cDNA of human TAP2 using an *in vitro* mutagenesis kit (In Vitro Mutagenesis system II; Amersham, UK) according to the manufacturer's instructions. The designed TAP2 mutant (*TAP2*Bky2*) was confirmed by direct sequencing. The cDNA for TAP2 and *TAP2*Bky2* were cloned into the *Not* I/*BamH* I site of the expression vector, pREP9 (G418 resistant). The cDNA for TAP1 was cloned to pREP7 (hygromycin resistant).

Cell lines and culture

The TAP-deficient cell line T2 (TxB cell hybridoma) was kindly provided by Dr. Takiguchi (The University of Tokyo, Tokyo, Japan). T2 cells were maintained at 37 C, 5% CO_2, in RPMI medium supplemented with 2 mM L-glutamine and 10% fetal calf serum (FCS). The pREP7-TAP1 containing the TAP1 gene and pREP9 containing TAP2B (pREP9-TAP2B) or *TAP2*Bky2* (pREP9-TAP2*Bky2) plasmid were doubly transfected to T2 by an electroporation method using a GenePulser™ (BioRad, Hercules, CA, USA). After transfection, the cells were selected in medium supplemented with G418 (1 mg/mL) and hygromycin (0.125 mg/mL). The stable transfectants positive for TAP1 and TAP2B, TAP1 and *TAP2*Bky2* were referred to as T2/B and T2/Bky2, respectively.

Flow cytometric analyses

One hundred thousand cells were incubated with an appropriate concentration of each primary antibody anti-MHC class I mAb (BB7.2: anti-HLA-A2 mAb or 4D12: anti-HLA-B5 mAb) or murine isotype control antibody, for 30 min on ice. Cells were then washed twice with cold phosphate-buffered saline (PBS) and additionally incubated with goat anti-mouse FITC Ig for 30 min on ice. Ten thousand viable cells were analyzed using a flow cytometer (FACSCalibur™; BD Biosciences, San Jose, CA, USA) and analyzed with CellQuest™ software (BD Biosciences). The expression levels for HLA-B5 and HLA-A2 were evaluated by mean fluorescence intensity (MFI).

Immunoprecipitation

A total of 1.5×10^9 cells were lysed with 1% CHAPS/TBS containing Complete Mini™ (Rosche, Mannheim, Germany) as a cocktail of protease inhibitors. Cell lysates were centrifuged at 15 000 rpm for 30 min. The supernatant from the T2 lysate was incubated with protein G Sepharose 4 Fast Flow™ (Amersham Biosciences AB, Uppsala, Sweden) conjugated with BB7.2 at 4 C overnight. For T2/B and T2/Bky2, supernatants were incubated with protein G conjugated with 4D12 overnight at 4 C, and then incubated with protein G conjugated with BB7.2. After three washes, the antibody-MHC complex was eluted with 0.2 M acetic acid. The eluate was filtered by a 5 kDa molecular cut-off filter. Immunoprecipitation was performed to produce three samples. For subsequent mass spectrometric analysis, triplicate samples were prepared from T2, T2/B and T2/Bky2.

Mass spectrometric analysis

MS/MS experiments were performed using a LCQ Deca ion trap instrument (ThermoQuest, San Jose, CA, USA) equipped with a Monitor C18 column (0.2 × 50 mm). The solvent system for on-line reversed-phase liquid chromatography was a linear gradient of solvent A mixed with solvent B, from 5% B to 60% B in 60 min. Solvent A was 0.1% aqueous formic acid and solvent B was 0.1% formic acid in acetonitrile. The flow rate was 1–2 μL/min. Spectra of eluting peptides were acquired in a data-dependent fashion by first acquiring a full MS scan from *m/z* 400–2000 followed by a MS/MS scan over the same scan range of the most intense ion recorded in the previous MS scan. MS/MS scans were acquired using an activation q_z of 0.250, activation time of 30 ms, and 35% normalized collision energy (for our LCQ Deca, 35% of full amplitude for *m/z* 1000 corresponds to 1.8 V). Protein identification was performed via a mass spectral database incorporating peptide mass and collision induced dissociation (CID) spectra, using MS-fit and MS-tag (SwissProt). Solvents were purchased from Nacalai Tesque (Kyoto, Japan).

RESULTS AND DISCUSSION

MHC class I complexes cannot be stably located on the cell surface unless antigenic peptides are loaded on MHC class I molecules. The genotypes of T2 cells for MHC class I are HLA-A0201 and B5101. However, HLA-B5 molecules were not detected on the surface of T2 cells by fluorescence-activated cell sorting (FACS) analysis (Fig. 1(a)) because of the instability of MHC class I molecules, since there is no peptide translation through the ER membrane via the TAP-dependent pathway. On the other hand, HLA-A2 was detected on the cell surface of T2 (Fig. 1(a)). HLA-A2 is known for its TAP-independent presentation of peptide LLDVTAAV on T2 cells.[12] This peptide was regarded as a signal sequence-derived peptide.[12] As shown in Figs. 1(b) and 1(c), we demonstrated that the co-expression of TAP1 and TAP2 brought HLA-B5 molecules to the cell surface. Furthermore, the MFI for HLA-A2 on T2/B and T2/Bky2 became stronger than on T2. This was probably due to the

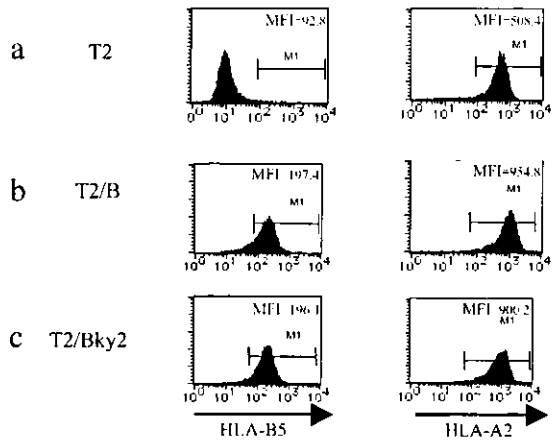

Figure 1. Flow cytometric analysis for HLA-B5 and HLA-A2 expression on T2, T2/B and T2/Bky2. (a) T2 did not express HLA-B5 but did express HLA-A2. (b, c) T2/B (double transfectant of TAP1 and TAP2B) and T2/Bky2 (double transfectant of TAP1 and *TAP2Bky2*) expressed HLA-B5 as well as HLA-A2. Furthermore, expression of HLA-A2 was upregulated on both T2/B and T2/Bky2.

stabilization of MHC molecules because TAP1 and TAP2 proteins generated by transfection carried the peptide across the ER membrane.

Three samples of each type were analyzed by mass spectrometry after purification by the cut-off membrane. After comparison of MS/MS spectra, we adopted the criterion that identification of peptides at least twice in three experiments is regarded as meaningful. Two peptides that were immunoprecipitated by BB7.2 mAb were common to T2, T2/B, and T2/Bky2. For example, we could detect similar peaks in mass spectra in the range *m/z* 898–899 from all three cell lines. MS/MS spectra of these mass peaks were the same, and turned out to correspond to LLDVTAAV in the database reference (Fig. 2), identical to the peptide reported previously by two groups.[12,13] We identified another peptide, LLSAEPVPA, derived from the CD 79b/Ig beta/B29 precursor, from T2 cells. This new peptide should also be presented on the cell surface with HLA-A2 via the TAP-independent pathway.

Seventeen peptides were common to T2/B and T2/Bky2, but not to T2. One of these peptides was IMLEALERV, based on the database reference (Fig. 3). Fourteen peptides were identified by immunoprecipitation with BB7.2 mAb (mAb to HLA-A2), and three peptides with 4D12 mAb (mAb to HLA-B5) (Table 1). More peptides were immunoprecipitated by BB7.2 than by 4D12. This may reflect the numbers of MHC class I molecules, since the MFI for HLA-A2 was four times higher than that of HLA-B5. All seventeen peptides matched with the presumed binding motif for HLA-A0201 or B5101. These results suggested that these peptides are probably the real peptides that are presented on the cell surface with MHC class I molecules via the TAP-dependent pathway. The peptide SLLGGDVVSV, derived from the hypothetical protein DFKZp 566A, has already been reported as one of

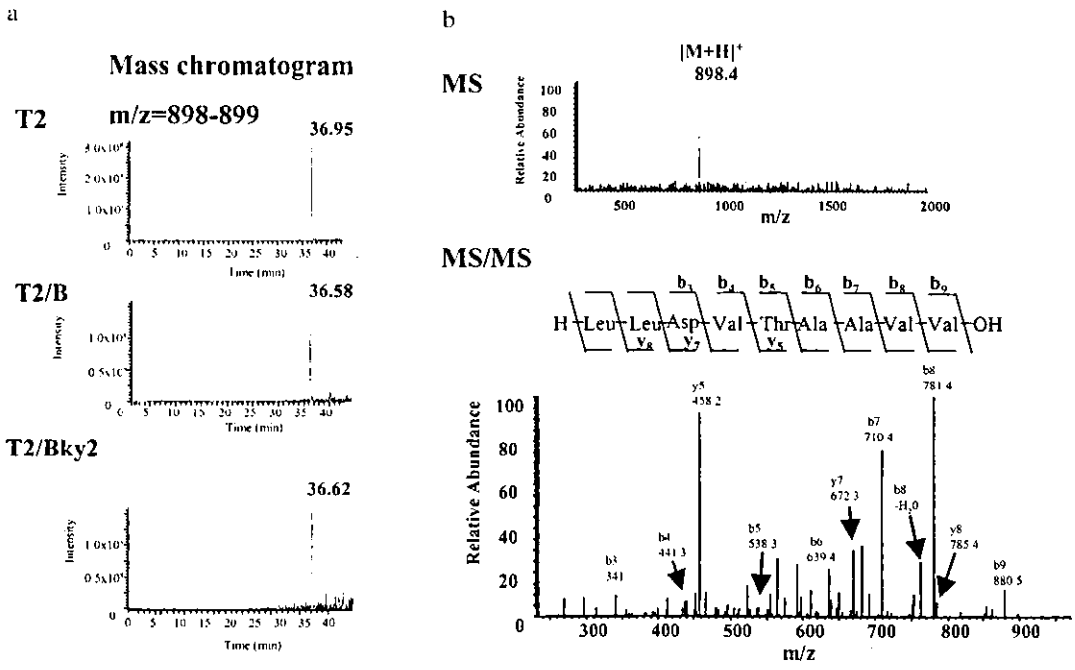

Figure 2. A typical HLA-A2 binding peptide common to T2, T2/B and T2/Bky2 detected by mass spectrometric analysis. (a) Mass chromatogram covering the *m/z* range 898–899 of the peptides from extracts of T2, T2/B and T2/Bky2. The chromatograms for T2, T2/B and T2/Bky2 showed this ion peak at retention times (RT) 36.95, 36.58 and 36.62 min, respectively. (b) MS and MS/MS spectra recorded at RT 36.95 min from extract of T2 cells. The y-series ions (C-terminal fragments), as well as those from b-series ions (N-terminal fragments), are shown. The MS/MS sequence, LLDVTAAV, matched a partial sequence of IFN-γ inducible protein. MS/MS spectra obtained for both T2/B at RT 36.58 min, and T2/Bky2 at RT 36.62 min, were essentially the same as that of T2.

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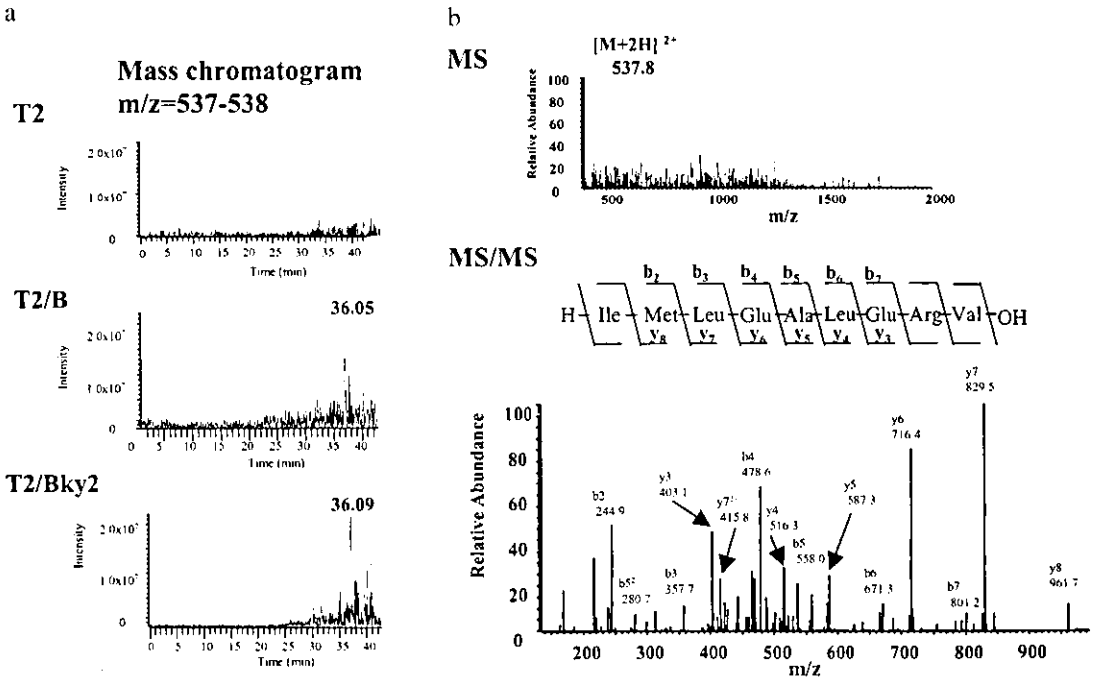

Figure 3. A typical HLA-A2 binding peptide common to T2/B and T2/Bky2. (a) Mass chromatogram covering the *m/z* range 537–538 of the peptides in extracts from T2, T2/B and T2/Bky2. The chromatograms for T2/B and T2/Bky2 showed this ion peak at RT 36.05 and 36.09 min, respectively, but that for T2 did not exhibit a peak in the RT range for *m/z* 537–538. (b) MS and MS/MS spectra of a peptide eluting at RT 36.05 min in the extract from T2/B cells. The spectrum shows the fragmentation pattern of the doubly charged precursor ion. The MS/MS sequence IMLEALERV matched the partial sequence of small nuclear ribonucleopolyprotein G. The MS/MS spectrum of T2/Bky2 recorded at 36.09 min was essentially the same as that of T2/B.

Table 1. List of peptides common to T2/B and T2/Bky2. (a) List of peptides recovered from HLA-A2 and (b) list of peptides recovered from HLA-B5. Ile or Leu was assigned to the peptide sequence based on identification by the SwissProt database

m/z	Sequence	Protein
a		
537.4	IMLEALERV	Small nuclear ribonucleopolypeptide G
500.5	SIIGRLLEV	Protein phosphatase 1 alpha catalytic subunit
536.4	ILDQKINEV	Ornithine decarboxylase 1
553.2	RLAVYIDRV	Lamin A/C
567.3	ILMEHIHKL	Ribosomal protein L19
786.6	SLAGGIIGV	Heterogeneous nuclear ribonucleoprotein K
896.5	GLYSGVTTV	Ribonucleotide reductase M1 polypeptide
903.4	GLATDVQTV	Proteasome subunit beta 3
936.4	ALLAGSEYL	Eukaryotic translation initiation factor 2
945.4	SLLGGDVVSV	Hypothetical protein DKFZp566A
960.3	ILTDITKGV	Eukaryotic translation elongation factor 2
974.4	MVDGTLLLL	HLA-E alpha chain precursor
1018.6	VMDSKIVQV	Importin alpha-1 subunit
1038.74	YLLPAIVHI	DEAD/H box polypeptide
b		
548.5	DAHIYLNHI	Thymidylate synthetase
509.5	DAYALNHTL	POU domain class 2
527.8	IPYHIVNIV	seryl-tRNA synthetase

the HLA A2-binding peptides in experiments using MCF7 and MDA-231 (breast cancer cell lines).[14] We identified two peptides that have been reported as autoantigens. An autoantibody against Lamin A/C has been reported in a patient with acute hepatitis triggered by the smooth muscle relaxant alverine.[15] The small nuclear ribonucleoprotein particle (snRNP) G is a component of the Sm core protein that is a common component of U1, U2, U4/6, and U5 RNP. Although the main epitopes for anti-Sm autoantibody belong to snRNP B/B′, D,[16] and E-F-G complex[17] in SLE patients, autoantibodies against snRNP G have been reported only in a few SLE patients.[18] The clinicopathological significance of the anti-snRNP G antibody has not been established, since the cross-reactivity between snRNP G and 70 kDa, one of the main epitopes for the anti-U1 RNP antibody, has been reported.[18] Therefore, we did not identify peptides strongly associated with autoimmune disease from peptides common to T2/B and T2/Bky2.

Moreover, we identified three peptides that were detected only in T2/Bky2: NLYPFVKTV (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICAR $TFase_{102-110}$), RMLPHAPGV (histone deacetylase (HDAC) 1 or $2_{372-380}$) and NQFPGFKEV (U1-snRNP $A_{229-237}$), by immunoprecipitation with BB7.2 mAb (Figs. 4(a), 4(b) and 4(c), Table 2). However, there was no peptide found only in T2/B. AICAR TFase is an enzyme responsible for catalysis of the ninth step in the *de novo* purine biosynthesis pathway. Methotrexate (MTX), the most popular anti-rheumatoid

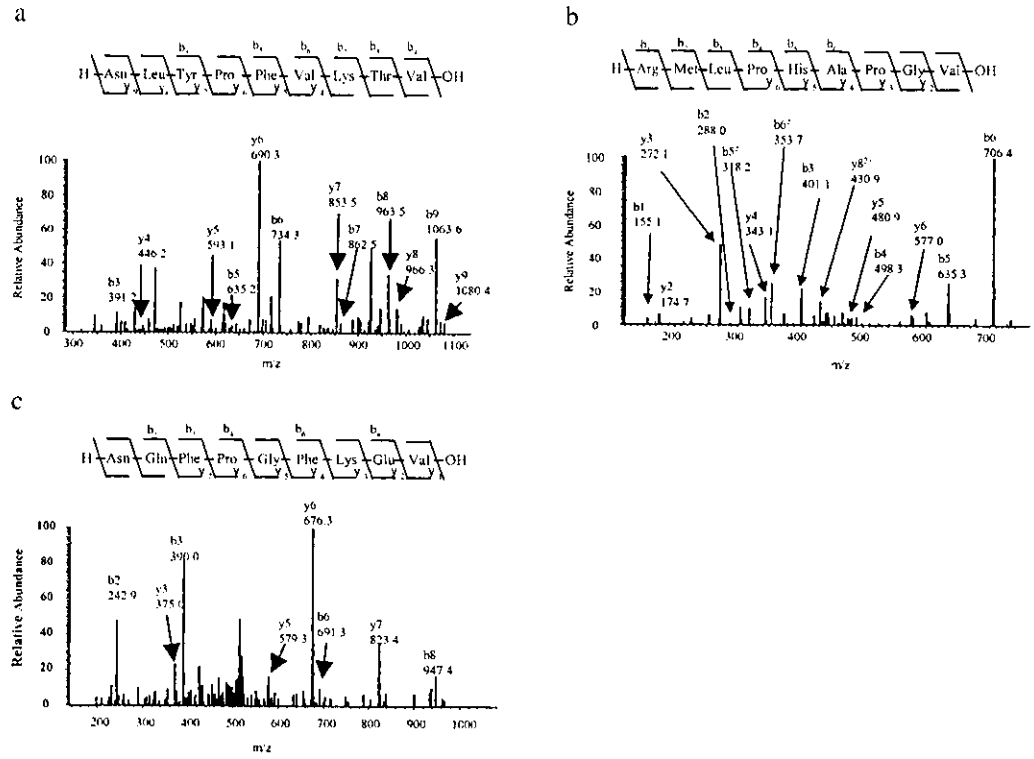

Figure 4. Peptides identified in extracts only from T2/Bky2. (a) The MS/MS sequence is NLYPFVKTV derived from 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase. (b) The MS/MS sequence is RMLPHAPGV derived from histone deacetylase 1 or 2. (c) The MS/MS sequence is NQFPGFKEV derived from small nuclear ribonucleopolypeptide A.

arthritis drug, inhibits AICAR TFase, which is considered to be one of the anti-rheumatic effects of MTX.[19] HDACs are key components in gene expression. In general, increased levels of histone acetylation (hyperacetylation) are associated with increased transcriptional activity, whereas decreased levels of acetylation (hypoacetylation) are associated with repression of gene expression. Therefore, HDAC inhibitors are anticipated to be anticancer agents.[20] Although the involvement of HDACs in autoimmune disease has not been demonstrated, HDAC may be related to the development of autoimmune disease. In fact, patients with certain connective tissue disorders have antihistone autoantibodies.[21,22] Furthermore, there have been reports that showed modulation of autoimmunity by HDAC inhibitors.[23,24]

U1 RNP is composed of 70 K, A, C, and Sm core protein. The snRNP A (U1A-RNP) is known to play a critical role in eukaryotic pre-mRNA splicing and polyadenylation. Some patients with SLE, mixed connective tissue disease (MCTD), or other autoimmune disorders, spontaneously produce autoantibody that exclusively precipitates U1 RNP *in vitro*. We have reported the association between *TAP2*Bky2* and anti-U1 RNP antibody in Japanese SLE patients.[9] The epitopes for anti-U1 RNP autoantibody are targeted 70K or A protein. The B cell epitopes for anti-A protein have been assigned to amino acids 165–185 and 232–256 of snRNP A.[25] The epitopes for auto-reactive CD4-positive T cells to U1 snRNP A were assigned to amino acids 209–228 and 262–281.[26] The peptide NQFPGFKEV (U1 snRNP $A_{229-237}$) overlapped the B cell epitope[25] and adjoined the epitope of CD4-positive T cells.[26]

The peptides we anticipated but failed to identify from T2/Bky2 cells were those from SS-A/Ro protein or other SS-related autoantigenic proteins such as SS-B/La or α-fodrin[27] or β-fodrin.[28] We cannot clearly address this question. One reason may be that thousands of different peptides are presented in vastly differing copy numbers per cell;[29] therefore, the peptides from SS-A/Ro antigen or other proteins on MHC class I may not be sufficiently abundant that we could detect them by mass spectrometry. Although we detected 22 peptides in all, the fact that we could detect three peptides that arose from only naturally mutated TAP2 transfectant cells is meaningful.

The mutated amino acid, Met to Val, of *TAP2*Bky2* at codon 577 is within NBD which is located in the cytosolic portion of

Table 2. List of peptides that were identified only in extracts from T2/Bky2

m/z	Sequence	Protein	Abbreviation	Position	Function
1081	NLYPFVKTV	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase	AICAR Tfase	102–110	Enzyme for catalysis of de *novo* purine biosynthetic pathway
489.4	RMLPHAPGV	Histone deacetylase 1 or 2	HDAC	372–380	Enzyme for histone deacetylation
533.5	NQFPGFKEV	Small nuclear ribonucleopolyprotein A	snRNP A	229–237	A component of U1 snRNP

TAP2. It has been assumed that the peptide and ATP/ADP independently bind to TAP in the cytosol, and the binding of peptide induces a structural reorganization of the TAP complex, triggering ATP hydrolysis and subsequent translocation of the peptide into the ER lumen.[8] Karttunen *et al.* have demonstrated that TAP2-mediated, but not TAP1-mediated, ATP hydrolysis is critical for peptide binding as well as translocation.[30] Therefore, the point mutation of NBD in TAP2 may play a critical role in the development of autoimmune diseases by transporting inappropriate peptides or not transporting appropriate peptides.

Taken together, to our knowledge this is the first report demonstrating that the peptides selectively loaded onto MHC class I are affected by the natural point mutation of TAP2. We believe that mass spectrometric analysis utilizing *TAP2*Bky2* mutants will contribute to better understanding of the development of autoimmune disease.

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N-Acetyltransferase 2 Genotype-Related Efficacy of Sulfasalazine in Patients with Rheumatoid Arthritis

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Purpose. For the individual optimization of drug therapy with sulfasalazine (SASP), we studied the influence of the *N-acetyltransferase 2* (*NAT2*) genotype on the pharmacokinetics, efficacy, and incidence of adverse reactions of SASP in patients.

Methods. Ninety-six rheumatoid arthritis (RA) patients were treated or had been treated with 0.5 and/or 1.0 g/day of SASP. The wild-type allele (*NAT2*4*) and three variant alleles (*NAT2*5B*, **6A*, and **7B*) of *NAT2* were determined by the polymerase chain reaction-restriction fragment length polymorphism method. Plasma concentrations of SASP and its two metabolites, sulfapyridine (SP) and *N*-acetylsulfapyridine (AcSP), were estimated by HPLC. Therapeutic efficacy and incidence of adverse reactions were also monitored as recommended by the American College of Rheumatology.

Results. Patients were classified into three groups by *NAT2* genotyping: Rapid Type (homozygote for *NAT2*4*), Intermediate Type (heterozygote for *NAT2*4* and variant alleles), and Slow Type (homozygote for variant alleles). There was no clear difference in the genotype frequencies between RA patients and healthy subjects. *NAT2* genotypes significantly affected both the plasma concentration ratios of SP to AcSP (SP/AcSP) and the efficacy of SASP ($p < 0.05$). Adverse reactions to SASP were found in 26 (27.1%) out of 96 patients, and there was no difference among the three genotype groups.

Conclusions. *NAT2* gene polymorphism is related to the plasma SP/AcSP ratio and the efficacy of SASP.

KEY WORDS: *N*-acetyltransferase 2; genotype; sulfasalazine; rheumatoid arthritis.

INTRODUCTION

Sulfasalazine (SASP), one of the disease-modifying antirheumatic drugs (DMARDs), has long been used in the treatment of rheumatoid arthritis (RA) (1). Many placebo-controlled studies have shown that SASP improves the erythrocyte sedimentation rate, duration of morning stiffness, pain (as assessed by visual analogue scale), articular index, number of swollen joints, number of painful joints, and patient's global assessment.

SASP is 5-aminosalicylic acid (5-ASA) linked by an azo bond to sulfapyridine (SP) (2). When orally administered, 30% of SASP is absorbed in the upper gastrointestinal tract, and the remainder is split in the colon by bacterial action into 5-ASA and SP (2,3). The 5-ASA remains largely within the large bowel, but the SP is totally absorbed and metabolized to N-acetylsulfapyridine (AcSP) predominantly by hepatic arylamine N-acetyltransferase 2 (NAT2). Both SASP and SP are considered to have a variety of actions, including immunomodulatory effects, antibacterial activity, and inhibition of folate-dependent enzymes (2–5). Adverse reactions such as nausea, vomiting, headache, malaise, hemolytic anemia and reticulocytosis appear to be dependent on the serum SP concentration (3,6–9). The *NAT2* gene exhibits a hereditarily determined polymorphism, and the individual phenotypes can be classified as rapid, intermediate, and slow acetylators by using isoniazid and sulfametazine as probe drugs (10). Several reports have described the relationship between the acetylator phenotypes and the pharmacokinetics, efficacy, and toxicity of SASP in the treatment of RA and inflammatory bowel diseases (IBD) (6–9,11–16).

In 1990, Deguchi *et al.* suggested that four *NAT2* alleles, including a wild-type allele (*allele1*) and three variant alleles (*alleles 2, 3,* and *4*) with a single nucleotide polymorphism (G857A, G590A, and C481T, respectively), could predict the acetylator phenotypes of isoniazid in healthy Japanese subjects (17,18). Then, we reported that individual acetylator phenotypes for procainamide and SASP in healthy subjects, and also for isoniazid in tuberculous patients, correlated with the combination of these four alleles (19–22). In addition, *NAT2* genotypes were shown to be associated with the capacity for SP acetylation and the incidence of adverse reactions in the SASP treatment of 13 patients with IBD (21).

Although several reports of the importance of acetylator phenotype or *NAT2* genotypes in SASP therapy have involved RA and IBD patients, there was no obvious conclusion concentrating the classification of subjects based on their *NAT2* genotype (11–14,21). To achieve this, further investigations of the relationships between the *NAT2* genotypes and

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ABBREVIATIONS: SASP, sulfasalazine; RA, rheumatoid arthritis; PCR-RFLP, polymerase chain reaction- restriction fragment length polymorphism; SP, sulfapyridine; AcSP, *N*-acetylsulfapyridine; DMARDs, disease-modifying antirheumatic drugs.

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efficacy or incidence of adverse reactions during SASP treatment were required in a large numbers of patients. In the present study, we studied the influence of the *NAT2* genotype on the pharmacokinetics, efficacy, and/or rate of adverse reactions of SASP in 96 patients with RA.

METHODS

Patients and Treatment

Ninety-six patients were randomly selected from medical records at five hospitals. All patients fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 criteria for RA and were being treated or had been treated with 0.5 g/day and/or 1.0 g/day of SASP in the form of enteric-coated Azulfizine® EN tablets (Pharmacia K. K., Tokyo, Japan). Nonsteroidal antiinflammatory drugs, prednisolone less than 7.5 mg/day, and methotrexate were coadministrated with SASP in the case of 69, 58, and 20 patients. Patients taking other DMARDs or immunosuppressive drugs were excluded from the study. Significant liver damage was not observed before treatment in all patients.

For 89 patients, the efficacy of SASP was determined by the physician's global assessment using a 6-scale grade according to the ACR criteria, and both grades 1 and 2 were considered to be effective (23). Adverse reactions were obtained from clinical symptoms and laboratory test data in the medical records. The aims of this study were fully explained to each patient who gave written informed consent. About 2 ml blood and plasma samples were stored below −20°C and sent to Kobe University Hospital. The following study protocol was approved by the Ethics Committee of each University.

NAT2 Genotyping

The most common mutations in the Japanese population at positions C481T, G590A, and G857A of the *NAT2* gene were determined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described previously (19,21,22). According to the nomenclature of the *NAT2* gene, wild-type and three variant alleles were defined as *NAT2*4* and **5B*, **6A*, **7B*.

Briefly, each genomic DNA was extracted from 0.5 ml whole blood using a DNA Extractor WB Kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). The three parts of the *NAT2* gene were amplified with a pair of primers for each, using a programmable heat block (Program Temp Control System PC-700, Astec Co., Fukuoka, Japan). To establish the presence or absence of each single nucleotide polymorphism, PCR products were digested with *Kpn* I, *Taq* I or *BamH* I, and the fragments were separated on 1.5%, 2%, or 2% agarose gels, respectively. Patients can be stratified by this genotyping into three groups: the homozygote for the wild-type allele *NAT2*4/*4* (named Rapid Type), the compound heterozygote for the wild-type and variant alleles *NAT2*4/*5B*, *NAT2*4/*6A*, and *NAT2*4/*7B* (named Intermediate Type), and the homozygotes for the variant alleles *NAT2*5B/*5B*, *NAT2*5B/*6A*, *NAT2*5B/*7B*, *NAT2*6A/*6A*, *NAT2*6A/*7B*, and *NAT2*7B/*7B* (named Slow Type). One hundred eighty healthy subjects also acted as controls for *NAT2* genotyping.

Determination of SASP, SP, and AcSP Concentrations

Twenty-seven blood samples (n = 17, 7, and 3 for Rapid, Intermediate, and Slow Types) were collected once 1–5 h after SASP administration and were immediately centrifuged at 3000 rpm (950 × *g*) for 5 min to separate the plasma, which was stored at −20°C until assay of the SASP, SP, and AcSP concentrations. It was confirmed that there was no alteration in the concentrations during freezing, and SP and AcSP concentrations in plasma were measured by HPLC (LC-10A series, Shimadzu Co., Kyoto, Japan) (21,22). The separation was carried out using a reversed-phase column (Nucleosil 10 C_{18}, 250 mm × 4.0 mm i.d., Chemco Chemical Co. Ltd., Osaka, Japan). The mobile phase was methanol/20 mM NaH_2PO_4 containing 20 mM tetra-*n*-butylammonium (30/70, vol/vol). The flow rate was 1.5 ml/min, and the column temperature was maintained at 40°C in a column oven (CT10A, Shimadzu). The calibration curves were linear over a concentration range of 0.5 to 100.0 μg/ml ($r^2 > 0.997$) for SASP, SP, and AcSP.

Statistical Analysis

Fisher's exact test and χ^2 test were used to compare the frequencies of efficacy and adverse reactions, and the Mann-Whitney *U* test was used to compare the other parameters among patient subgroups.

RESULTS

NAT2 Genotype

The frequencies of the *NAT2* genotypes in 96 Japanese RA patients and 180 healthy Japanese subjects are shown in Table I. The allele frequencies of *NAT2*4*, *NAT2*5B*, *NAT2*6A*, and *NAT2*7B* were 73.5, 0.0, 20.8, and 5.7% in RA patients and 71.1, 1.9, 18.1, and 8.9% in healthy subjects, respectively. The genotype and allele frequencies of the *NAT2* gene were not significantly different between the groups. The frequencies of the three *NAT2* genotype groups, Rapid Type, Intermediate Type, and Slow Type, in RA patients (55.2, 36.4, and 8.3%) were also similar to those in healthy subjects (48.3, 45.5, and 6.2%, respectively).

Table I. Frequencies of *NAT2* Genotypes in 96 Patients with RA and 180 Healthy Subjects

NAT2 genotype		Number (%)	
Group	Genotype	RA patients	Healthy subjects
Rapid	*NAT2*4/*4*	53 (55.2)	87 (48.3)
Intermediate	*NAT2*4/*5B*	0 (0.0)	6 (3.3)
Intermediate	*NAT2*4/*6A*	27 (28.1)	54 (30.0)
Intermediate	*NAT2*4/*7B*	8 (8.3)	22 (12.2)
Slow	*NAT2*5B/*7B*	0 (0.0)	1 (0.6)
Slow	*NAT2*6A/*6A*	5 (5.2)	3 (1.7)
Slow	*NAT2*6A/*7B*	3 (3.1)	5 (2.8)
Slow	*NAT2*7B/*7B*	0 (0.0)	2 (1.1)
	Total	96 (100)	180 (100)

Plasma Concentrations of SASP, SP, and AcSP

Figure 1 shows the plasma concentrations of SASP, SP, and AcSP obtained 1–5 h after SASP administration in 27 RA patients. In each *NAT2* genotype group, these plasma concentrations exhibited substantial interindividual variation. Plasma concentration ratios of SP/AcSP less than 0.2 were seen in 82.4% of the Rapid Types and in 42.9% of the Intermediate Types; however, the figure for the Slow Types was 0.0% (Fig. 2). In contrast, plasma SP/AcSP ratios over 0.4 were seen in 0.0% of the Rapid Types, 28.6% of the Intermediate Types, and 33.3% of the Slow Types. Factorial analysis by the variance method showed that the *NAT2* genotype affected the plasma SP/AcSP ratios significantly ($p = 0.049$); however, the dose of SASP had no effect ($p = 0.6847$).

Efficacy

Patient profiles of each *NAT2* genotype group are shown in Table II. Sex, disease duration, and SASP dose were not significantly different in the three groups. The duration of SASP treatment in the Slow Types was much shorter compared with the other two types ($p < 0.05$ by Mann-Whitney *U* test). In Table III, the physicians' global assessment showed that SASP was effective in 48 of 89 patients (53.9%). In each genotype group, all patients who were Slow Types benefited, and this was significantly higher than in the case of the Rapid Types ($p < 0.01$) or Rapid Types plus Intermediate Types ($p < 0.05$).

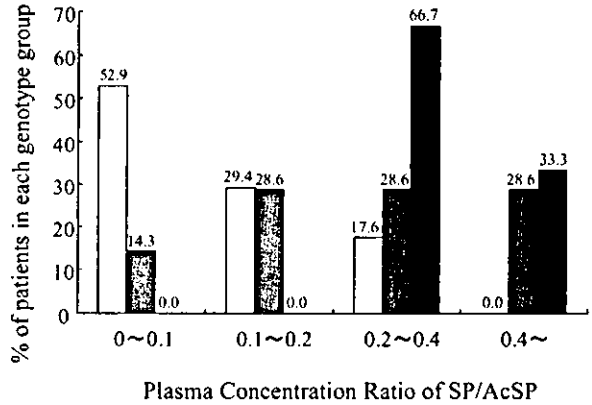

Fig. 2. Plasma concentration ratio of SP/AcSP from 1 to 5 h after SASP administration in three groups classified according to the *NAT2* genotypes: 17 Rapid Types (open bars), 7 Intermediate Types (gray bars), and 3 Slow Types (solid bars). Each column shows the percentage of patients in each genotype group.

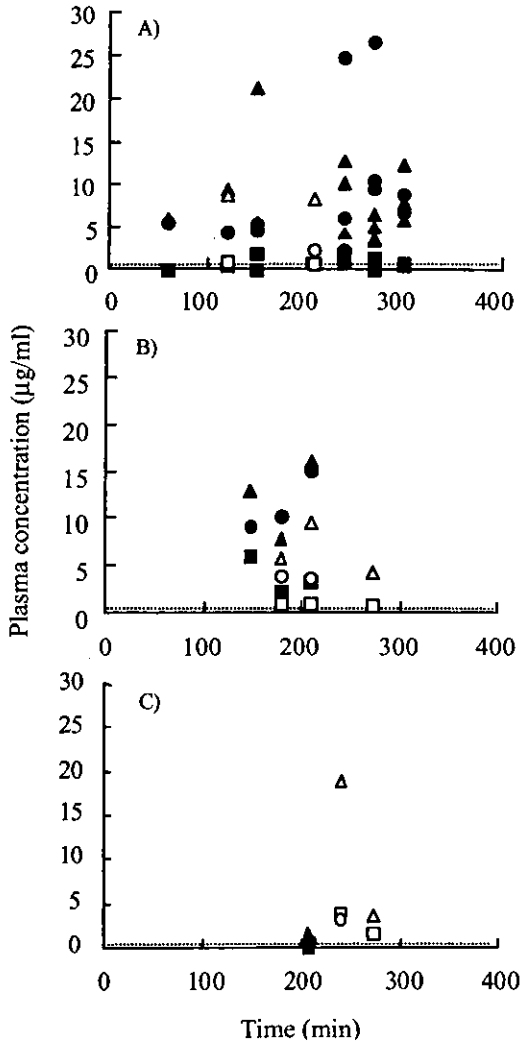

Fig. 1. Plasma concentrations of SASP (circles), SP (squares), and AcSP (triangles) after SASP administration in three groups classified according to *NAT2* genotypes: 17 Rapid Types (A), 7 Intermediate Types (B), and 3 Slow Types (C). Doses of SASP, 0.5 g/day and 1.0 g/day, are shown by open and closed symbols, respectively. Dotted lines (0.5 μg/ml) show the lower limits of SASP, SP, and AcSP.

Adverse Reactions

The total number of adverse reactions found in 96 patients was 34 (Table IV). Adverse reactions associated with SASP were found in 26 patients (27.1%), and the patients withdrawing from treatment because of adverse reactions were as follows: seven Rapid Types (13.2%), two Intermediate Types (5.7%), and two Slow Types (25.0%). Cutaneous symptoms (skin rash and/or itching) occurred in seven Rapid Types (13.2%), eight Intermediate Types (22.8%), and one Slow Type (12.5%). In contrast, gastrointestinal symptoms (nausea and/or vomiting) occurred in three Rapid Types (5.7%), three Intermediate Types (8.6%), and one Slow Type (12.5%). Systemic hypersensitivity with high fever, dyspnea, and decreased consciousness level was seen in one Rapid Type patient. The ratio of each adverse reaction was not significantly different among the three groups. Combination with methotrexate and/or prednisolone did not affect the occurrence of adverse reactions.

DISCUSSION

More than 50 years ago, NAT2 was first found to exhibit polymorphisms in several hepatic drug-metabolizing enzymes (24,25). Then, the phenotyping method using probe drugs was developed, and this led to studies on the relationships between the acetylator phenotypes and pharmacokinetics, efficacy, and/or toxicity. To date, at least 17 mutant alleles have been found in the human *NAT2* gene; however, genotyping of three *NAT2* point mutations was sufficient to predict the metabolism of INH in Japanese tuberculous patients as well as healthy subjects (20). Compared with phenotyping, genotyp-

Table II. Patient Profiles of Three Groups Classified According to the *NAT2* Genotypes

	NAT2 genotype			
	Total	Rapid *NAT2*4/*4*	Intermediate *NAT2*4/*6A* *NAT2*4/*7B*	Slow *NAT2*6A/*6A* *NAT2*6A/*7B*
Number of patients	96	53	35	8
Male/female	20/76	13/40	5/30	2/6
Age (year)	58.0 ± 12.7	57.6 ± 13.2	58.6 ± 12.0	58.3 ± 13.7
Disease duration (year)	7.7 ± 6.4	6.3 ± 4.7	9.8 ± 8.3	8.4 ± 4.7
SASP 0.5 (g/day)/1.0 (g/day)	26/70	14/39	9/26	4/4
Duration of treatment (month)	13.1 ± 8.4	12.4 ± 8.0	15.8 ± 8.4	5.8 ± 4.6#
PSL treatment	58	31	22	6

# $p < 0.05$ compared with the Rapid Type and Intermediate Type by the Mann-Whitney *U* test.

ing is a simple and rapid technique and more reliable for typing patients with renal, hepatic, and gastrointestinal disorders. However, the relationships between the *NAT2* genotypes and pharmacokinetics, efficacy, or toxicity have not been studied in detail in the case of SASP therapy.

Both genotype and allele frequencies of the *NAT2* gene were not significantly different between RA patients and healthy subjects (Table I). Some data have indicated that acetylation influences the process of inactivation of excessive biogenic amines, including histamine, which is responsible for allergic reaction symptoms (26–28). Moreover, some research has suggested that the Slow Type is an important factor in the individual susceptibility to rheumatoid arthritis (29). However, our results show that acetylator status is not involved in the onset of RA.

In spite of the classification of *NAT2* genotypes, the plasma concentrations of SASP, SP, and AcSP (1–5 h) exhibited substantial interindividual variations (Fig. 1), which might be caused by poor (10–30%) and slow absorption of SASP (15). In contrast, the acetylation index (10), the plasma SP/AcSP ratio, correlated with the *NAT2* genotypes (Fig. 2). The period of 1–5 h represents the elimination phase of both SP and AcSP because the mean absorption lag time of SP is 6 h, and the mean time to reach the maximum plasma concentrations of SASP and SP are 6 and 12–14 h, after the multiple administration of 1.0 g SASP in healthy Japanese subjects (30). The pharmacokinetics of SASP is dose linear in the dose range of 0.5 to 2.0 g following a single administration in a healthy subject. Also, in our previous study with healthy subjects, the plasma SP/AcSP ratios of the Rapid Types [0.24 (mean value; n = 4)] were significantly lower than the Intermediate Types [0.53 (n = 3)] at 24 h after multiple administration of 1.0 g SASP (22). Therefore, the plasma SP/AcSP ratio was affected by the *NAT2* genotype and not by the dose of SASP, coadministered drugs, coexisting diseases, age, etc.

Although several reports of the dependence of acetylator phenotype or *NAT2* genotypes in the SASP therapy have involved RA and IBD patients, no conclusion was reached concentrating the classification of the subjects based on their *NAT2* genotype (11–14,21). In the present study, 0.5 and/or 1.0 g/day of SASP was administered to RA patients, which was lower than other previous reports (1.0–3.0 g/day) (12–14). As a result, the physicians' global assessment showed that Slow Types (100.0%) benefited more than Intermediate Types (63.6%) and Rapid Types (40.8%) (Table III). It should be noted that the efficacy of SASP treatment was more effective in Slow Types, although the number of subjects was small. This seems to be consistent with the shorter duration of SASP treatment in Slow Types compared with the other two Types. These findings suggest that the *NAT2* genotype is an important factor in determining the efficacy of SASP in RA patients.

Table III. Efficacy of SASP in Three Groups Classified According to the *NAT2* Genotypes

	NAT2 genotype			
	Total n = 89	Rapid *NAT2*4/*4* n = 49	Intermediate *NAT2*4/*6A* *NAT2*4/*7B* n = 33	Slow *NAT2*6A/*6A* *NAT2*6A/*7B* n = 7
0.5 g/day				
Grade 1	1/24	0/13	1/8	0/3
Grade 2	13/24	5/13	5/8	3/3
Subtotal	14/24 (58.3%)	5/13 (38.5%)	6/8 (75.0%)	3/3 (100.0%)
1.0 g/day				
Grade 1	8/65	4/36	2/25	2/4
Grade 2	26/65	11/36	13/25	2/4
Subtotal	34/65 (52.3%)	15/36 (41.7%)	15/25 (60.0%)	4/4# (100.0%)
Total	48/89 (53.9%)	20/49 (40.8%)	21/33 (63.6%)	7/7##,* (100.0%)

# $p < 0.05$ and ## $p < 0.01$ compared with the Rapid Type by Fischer's exact test.
* $p < 0.05$ compared with the Rapid Type + Intermediate Type by Fischer's exact test.

Table IV. Adverse Reactions of SASP in Three Groups Classified According to the *NAT2* Genotypes

	NAT2 genotype			
	Total (n = 96)	Rapid (n = 53)	Intermediate (n = 35)	Slow (n = 8)
Patient number of adverse reactions				
0.5 g/day SASP	11/26	4/14	6/9	1/4
1.0 g/day SASP	15/70	9/39	5/26	1/4
Total	26 (27.1%)	13 (24.5%)	11 (31.4%)	2 (25.0%)
Withdrawal because of toxicity	11 (11.5%)	7 (13.2%)	2 (5.7%)	2 (25.0%)
Adverse reactions				
Cutaneous#	16 [6]	7 [4]	8 [1]	1 [1]
Gastrointestinal##	7 [3]	3 [1]	3 [1]	1 [1]
Elevated liver enzymes	3 [1]	3 [1]	0	0
Hematological###	1	1	0	0
Cardiovascular####	2	0	1	1
Systemic hypersensitivity	1 [1]	1 [1]	0	0
Others	4	1*	3**	0

Rash and/or itching; ##nausea and/or vomiting; ###leucopenia; ####palpitation.
[ ] = numbers of patients withdrawn.
* Anemia; **dry mouth, ischemic heart disease, or diabetes mellitus.

In general, the adverse reactions of SASP were divided into two groups. One involves hypersensitive reactions, such as skin rash, aplastic anemia, and hepatic and pulmonary dysfunctions, which are independent of the dose of SASP or blood concentration (31). The other adverse reactions are dependent on the dose of SASP or blood concentration of SP (> 50 μg/ml), such as nausea, vomiting, headache, malaise, hemolytic anemia, and reticulocytosis (6). In the phenotyping study, a higher incidence of nausea/vomiting and raised hepatic enzymes was seen in slow acetylators (14). As far as our results are concerned, no correlation between the *NAT2* genotype and the overall toxic reactions of SASP was seen, although the rate exhibited a trend: Rapid Type < Intermediate Type < Slow Type (Table IV). This discrepancy may be explained by the low incidence of adverse reactions caused by the low dose (0.5 and/or 1.0 g/day) of SASP given in the form of enteric-coated tablets. Small numbers of the Slow Type, 8 out of 96 patients, make it difficult to evaluate the influence of the *NAT2* genotype on toxicity. As in previous reports, we observed hypersensitive reactions in all genotype groups with the same incidence.

In summary, we conducted the *NAT2* genotyping of wild-type and three variant alleles in Japanese RA patients. The *NAT2* gene polymorphism has no influence on the onset of RA or on the incidence of adverse reactions to SASP, although there was an effect on the plasma SP/AcSP ratio and the efficacy of SASP. These data suggest that *NAT2* genotyping could become a useful alternative to therapeutic drug monitoring for SASP. A further prospective study will be required to determine the effective and safe dosage regimen of SASP for RA, which can be decided based on the *NAT2* genotype of individual patients.

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