さらに同様の実験系で補中益気湯についても検討を行ない、桂皮と同様のウイルス感染抑制効果を補中益気湯に認めている（**図3**）。これについて服部氏は補中益気湯の構成成分である柴胡・黄蓍が特に抗ウイルス作用に関与している可能性を指摘している。

「ウイルス量はHIV感染予後因子にあらず」の衝撃

もう一つの論文は、今年のJAMA 9月27日号に載った「Predictive Value of Plasma HIV RNA Level on Rate of CD4 T-Cell Decline in Untreated HIV Infection」である[3]。これは、1984-2004年間に行なわれた約3000人対象のコホートスタディで明らかになった、HIV感染者の未治療段階での血清ウイルス量はその後のAIDS発症の予後予測因子にはならない、という解析結果である。この論文に対し、かつてHIV感染者のウイルス量がAIDS発症と生命予後を規定するとの論文を掲載したScienceが9月29日号でコメントを発表したのをみても、その論文の衝撃の大きさがうかがえる。JAMA掲載の論文の考察では、CD4T細胞の減少を規定するのはウイルス量ではなく他の因子-まだ確定はできないがおそらくは長期間の炎症持続状態の関与を示唆している。

服部氏はこの論文について「ウイルス量と予後は必ずしも一致しないということは、多くの臨床家が感じていたことで、その間を埋める因子として、全身性炎症の持続が指摘されたことになる」と評価。慢性ウイルス感染におけるバイオディフェンスを考えるうえで、炎症反応という新たなターゲットが提示されたのである。

全身性炎症抑制作用を補中益気湯で確認

全身性炎症の持続については、一見ウイルス感染とは関係のない領域でも興味深い報告がある。

その一つが、慢性閉塞性肺疾患（COPD）を全身性炎症としてとらえ、その対策として補中益気湯の有用性を検討した千葉大学の巽氏の研究である。

かつてのCOPDの疾患概念は、気道炎症と肺胞破壊という局所病変を背景にした、進行性で非可逆性の一秒量低下を病態の本質としてとらえるものだった。しかし、2004年にCelliにより、%FEV1・BMI・運動能力・運動時の息切れの4項目を指標としたBODE indexがCOPD患者の予後予測因子となることが報告[4]された頃から、COPDは全身性疾患として認識されるようになり、特に栄養状態と予後の関係が注目されるようになってきた。

巽氏は、COPD患者では気道炎症反応で生じた炎症性サイトカインが他組織におよび、全身性の炎症反応を惹

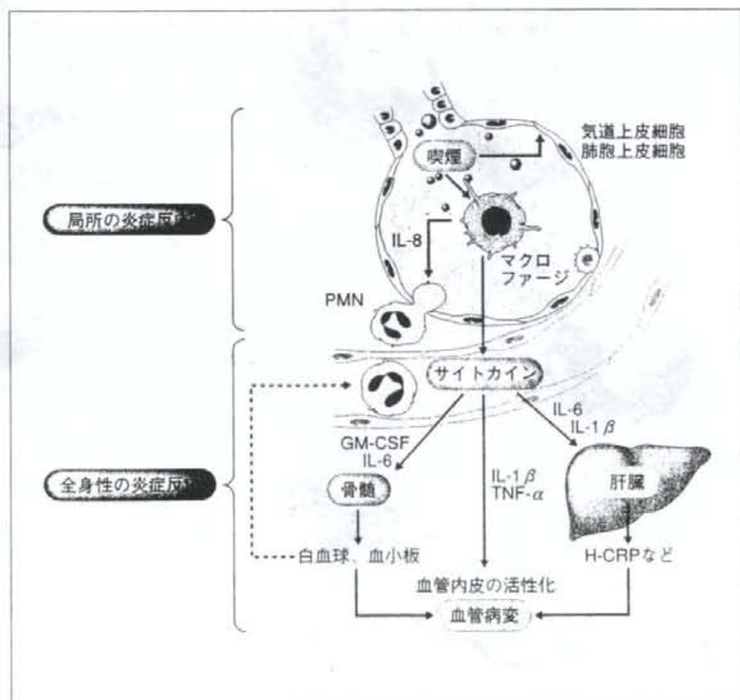


図4　COPDと全身性炎症

起しているとの考えに立ち（図4）、呼吸器疾患症状（SGRQのSymptom score）、漢方医学的な体調評価（気虚スコア）、感冒回数、急性増悪回数、炎症指標[5]（高感度CRP、TNF-α、IL-6）、栄養指標（プレアルブミン、レプチン）、動脈硬化指標（高分子量アディポネクチン）を評価項目として、COPD患者に対する補中益気湯の効果を調べた。対象はCOPD症例35例で、うち17例に補中益気湯を投与し、18例を対照群とした。その結果、補中益気湯投与群ではSGRQのSymptom scoreおよび気虚スコアが改善し、感冒罹患頻度、急性増悪頻度が抑制され（図5）、高感度CRPおよびTNF-α値の低下が認められた。また、%一秒量と各種炎症指標とは負の相関がみられ、COPDが重症化するほど全身性炎症反応が強まっていることが示唆された。こうした結果は、COPDの病態は栄養状態および全身性炎症反応と深く関与し、補中益気湯はCOPDの栄養状態を改善し、全身性炎症反応を抑制している可能性を示唆するものといえる。

また、動脈硬化指標のアディポネクチン値も補中益気湯群で改善が認められた。これについて巽氏は、「近年、動脈硬化を血管壁の炎症反応としてとらえるようになり、また欧米ではCOPD患者に心血管イベントの発症頻度が高いという指摘がある。そういう意味でもCOPDを全身性炎症反応とする考え方は示唆に富んでいる」と、炎症反応に関与している可能性を指摘した。

過剰な炎症を抑え、免疫抑制を是正

このように、全身性炎症反応の抑制効果が指摘された補中益気湯だが、その一方で従来からNK細胞の活性化やCTL免疫誘導といった免疫賦活作用も知られている（図6）。

ここで一つの疑問が生じる。一般に免疫応答は炎症反応をともなうものである。果たして補中益気湯の抗炎症作用と免疫賦活作用は両立するものなのだろうか。

これについては、岡山大学の岩垣氏の周術期患者に補中益気湯を投与したデータが一つの答えを示している。進行胃・大腸癌患者を対象とし、補中益気湯の術前投与が術後の全身性炎症反応症候群（SIRS）とそれに引き続き起こる代償性抗炎症反応症候群（CARS）への影響をみたもので、これによると、補中益気湯群では手術後の体温上昇が非投与群に対し抑えられ、術後感染症併発率（術後予防投与に用いた抗菌薬とは異なる抗菌薬を術後2週間以内に投与した症例を術後感染症併発と定義）も補中益気湯群で有意

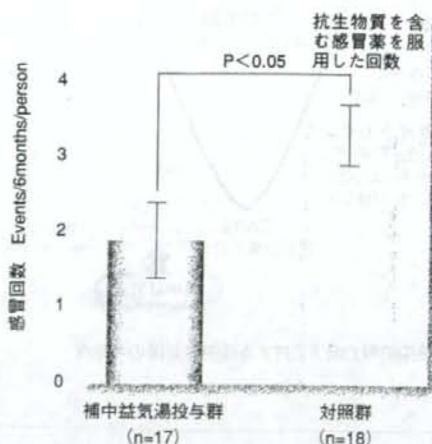


図5　COPD患者に対する補中益気湯投与群と対照群での感冒罹患頻度

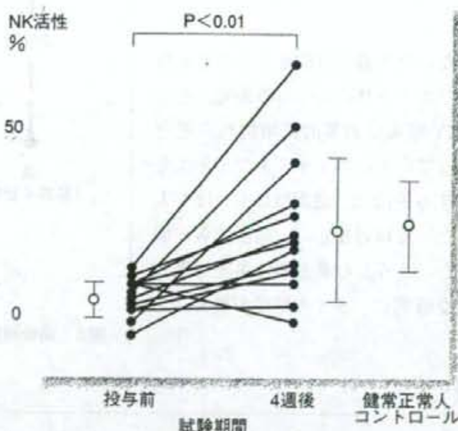


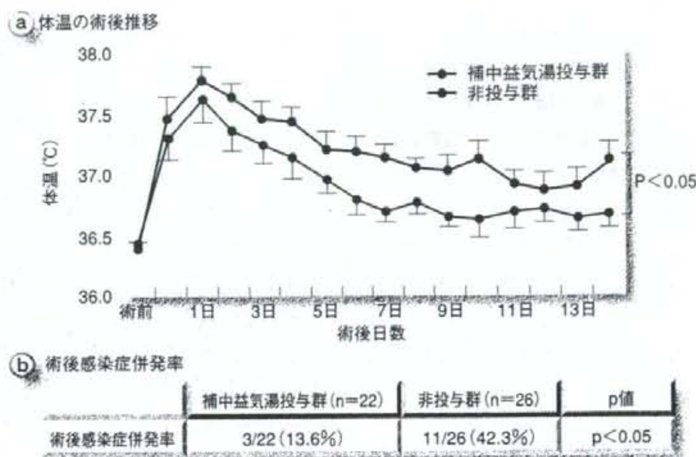
図6　NK活性低値例における補中益気湯の効果

に減少していた(**図7**)。このデータは、補中益気湯が術後の過度の炎症反応および免疫低下をどちらも改善していることを示すものといえる(**図8**)。

こうした補中益気湯がもつ作用の多面性について服部氏はこう述べる。「漢方は体内の環境バランスを整える方向で作用します。補中益気湯が免疫賦活作用・抗ウイルス作用・抗炎症作用を併せもつことを示すこれらのデータは、同剤が生体防御に有利な状態に体内環境を整える方剤であることを示しているといえます」。

ここで紹介したデータの他にも、補中益気湯についてはインフルエンザ感染マウスの生存率向上、感冒(ライノウイルス)感染抑制、ヘルペスウイルス感染抑制、タバコ刺激に対する抗炎症効果、などの報告もある。こうした作用は同剤の多面的作用によるバイオディフェンス増強作用によるものといえるだろう。

ひたひたと着実に広がりを見せる鳥インフルエンザの人への感染例、そしてHIV感染は世界的に増加の一途をたどっている。バイオディフェンスを増強する手段は、選択肢は多いほど人類にとっては好ましい。服部氏らが取り組む、こうした漢方薬の薬理効果の新たな解明に、多くの期待が集まっている。



	補中益気湯投与群(n=22)	非投与群(n=26)	p値
術後感染症併発率	3/22(13.6%)	11/26(42.3%)	p<0.05

図7　体温の術後推移と術後感染症併発率に対する補中益気湯の効果

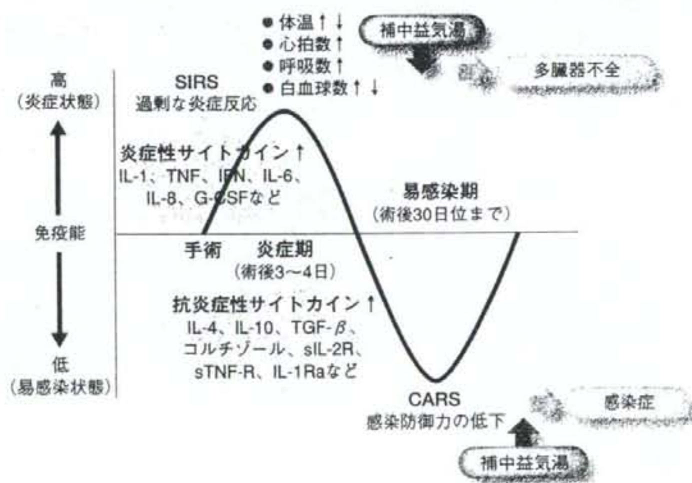


図8　術後侵襲による炎症反応・感染防御力低下に対する補中益気湯の可能性

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※図7は別刷制作時に追記したものです。

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Competitive Study of Monoclonal Antibodies against the HIV-1 Gp41 Core Structure

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***Abstract*: Monoclonal antibodies (MAbs) 50.69, 98.6, and T26 bind specifically to the core structure of the human immunodeficiency virus type 1 (HIV-1) envelope transmembrane glycoprotein (gp41). To clarify the specificity of the anti-core structure MAbs, we performed competitive assays using the MAbs to the H9 human T cell line infected with the IIIB strain of HIV-1 (H9/IIIB). Bound MAb 50.69 inhibited MAb 98.6 binding unidirectionally. The reason for the unidirectional cross competition between MAbs 50.69 and 98.6 is not clear, but these results help to define the antigenic structure of gp41 on the surface of infected cells.**

***Key words*: HIV-1, Gp41, 50.69 antibody, 98.6 antibody**

The binding of envelope glycoprotein 120 (gp120) of human immunodeficiency virus type 1 (HIV-1) to cellular receptors on target cells leads to conformational changes of envelope transmembrane glycoprotein (gp41) that permit viral and cellular membrane fusion (12). Recent crystallographic studies have shown that fusion-active gp41 folds into a six-helix α-helical bundle, in which three N-terminal helices (N peptides) form an interior, parallel-coiled-coil trimer, while three C-terminal helices (C peptides) pack in the reverse direction into three hydrophobic grooves on the surface of this coiled coil (2, 9, 16). We have previously reported that the decline of anti-DP107 (aa 553–590) (α-helical N-peptide) antibody is associated with the clinical progression in HIV-1-infected individuals, suggesting that antibodies against the structure may have a protective role (7). To define the epitopes within these regions to which infected humans respond during the course of infection, the specificity of human MAbs to these regions was studied. Using 10 human MAbs identified initially by their reactivity to whole gp41 in HIV-1 virion lysates, Xu et al. previously reported two immunodominant regions of gp41 that define the epitopes within these regions to which infected humans respond during the course of infection (5, 17). The first region of gp41 is in the vicinity of the cysteines between amino acids 598 and 604 (cluster I). The second immunogenic region position is between 644 and 663 (cluster II). Titration of sera from HIV-1-infected patients showed that there was approximately 100-fold more efficient antibody binding to cluster I than to cluster II in patients' sera, confirming the immunodominance of cluster I. Subsequent studies have disclosed that human MAbs against gp41 could recognize the gp41 core structure (14). We called these MAbs anti-core structure MAbs, because the exact antigenic structure of gp41 clarified so far is only the core structure and the native and fusion-active structure of all gp41 molecules has not been clarified yet. MAb 50.69, which is a cluster I MAb, reacts to a N51 (aa 540–590) and C43 (aa 624–666) peptide mixture (N51/C43) (6), but neither to N36 (aa 546–581)/C34 (aa 628–661) nor a single C43 peptide (17). MAb 98.6, which is a cluster II MAb, reacts to N51/C43, N36/C34, and a C43 peptide (14). Both N51/C43 and N36/C34 are known to form a six-helical bundle (2). MAb T26 was reported to bind to the six-helix bundle N36/C34 (1, 3, 4). These studies

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FACS, flow cytometry; gp41, envelope transmembrane glycoprotein 41; gp120, envelope glycoprotein 120; gp160, envelope glycoprotein 160; HIV-1, human immunodeficiency virus type 1; H9/IIIB, the H9 human T cell line infected with the IIIB strain of HIV-1; MAbs, monoclonal antibodies; MFI, mean fluorescence intensity; PE-avidin, phycoerythrin-labeled avidin.

suggest that MAb T26 is specific for the core structure because MAb T26 was reported to bind only to the oligomeric form in immunoprecipitation assays. Therefore, these MAbs are expected to bind to different epitopes. These MAbs must bind to the infected cell surface gp41 in the human body, although it is not known whether these MAbs interfere with the bindings of other MAbs. To clarify this issue we did competitive assays among human MAbs 50.69, 98.6 and T26.

One half million H9/IIIB cells, a human T cell line infected with the IIIB strain of HIV-1, were pre-incubated with saturated concentrations of MAbs 50.69, 98.6 or human IgG (Calbiochem, La Jolla, Calif., U.S.A.) for 1 hr at 4 C, followed by washing. The effects of these MAbs on the binding of biotinylated MAbs (B-50.69, B-98.6 and B-T26) to H9/IIIB cells were studied. Biotinylated MAbs 50.69 and 98.6 were provided by Dr. Miroslaw K. Gorny. MAb T26 IgG was purified from culture supernatants of hybridoma cells obtained from Dr. P.L. Earl and biotinylated. Because MAb T26 bound little at 4 C, we did not use MAb T26 as a competing MAb. B-50.69 (2 μg/ml), B-98.6 (2 μg/ml) or B-T26 (32 μg/ml) at saturated concentrations were added to the cells and incubated for 30 min at 4 C for MAb B-50.69 or MAb B-98.6, and for 15 min at 37 C for B-T26. After washing the cells twice, 4 μl phycoerythrin-labeled avidin (PE-avidin) (Serotec, Ltd., Kidlington, Oxford, U.K.) was added and the cells were incubated for 30 min at 4 C followed by fixation. PE-avidin labeled biotinylated MAb was detected by flow cytometry (FACS).

Since we could not detect efficient MAb T26 binding to H9/IIIB at 4 C, we tried to detect the temperature dependency of the MAb T26 binding. MAb T26 bound H9/IIIB and yielded a low mean fluorescence intensity (MFI) at 4 C, and a high MFI at 37 C (Fig. 1). The saturating concentrations were 64 μg/ml at 4 C and 32 μg/ml at 37 C and the saturated MFI at 4 C was 11.5 and significantly lower than the saturated MFI 18.8 at 37 C ($P<0.0167$).

We evaluated the MAb T26 binding at 37 C, and those of MAbs 50.69 and 98.6 at 4 C, and calculated the % inhibition (Fig. 2). MAb 50.69 pre-incubation blocked its own binding as a positive control. MAb 98.6 did not block MAb B-50.69 binding ($P\geqq0.05$). MAb B-98.6 was also examined in a similar condition. MAb 98.6 pre-incubation blocked MAb B-98.6 binding. The MAb 50.69 pre-incubation blocked MAb B-98.6 binding ($P<0.0167$), though the former MAb inhibited the binding more efficiently than the latter did. We were able to observe that MAbs 50.69 and 98.6 bound at 4 C and avoided non-specific binding, although the condition was not physiological (13). We also confirmed that MAb B-T26 binding at 37 C was blocked by bound MAb T26. The MAb 50.69 pre-incubation did not show a significant blocking effect for MAb B-T26 ($P\geqq0.05$). However, pre-incubation of MAb 98.6 blocked its binding very efficiently ($P<0.0167$).

MAb T26 was identified as an anti-gp41 antibody that binds only to oligomers, particularly to trimers, but not to monomers (4). Both MAbs 98.6 and T26 bind to a mixture of N36/C34, but their precise epitopes must be different from each other because MAb 98.6 also binds to monomeric gp41. MAb 98.6 pre-incubation inhibited MAb B-T26 binding to H9/IIIB, indicating the epitopes of MAbs 98.6 and T26 may partially overlap.

On the other hand, MAb 50.69 did not compete with MAb B-T26 at all, indicating that the epitopes of the two MAbs are distant. These data accord with the findings that MAb 50.69 does not bind to a mixture of N36/C34 but MAb T26 does.

MAb 98.6 binds to a unique epitope shared with a mixture of N51/C43, N36/C34 and C43. But MAb 50.69 binds specifically to a mixture of N51/C43 but not C43. It is worthwhile to note that MAb 50.69 pre-incubation inhibited MAb 98.6 binding but the interference was not reciprocal. As shown previously, MAbs 50.69 and 98.6 did not compete in an enzyme-linked immunosorbent assay (ELISA) using viral lysate or recombinant proteins derived from gp41 (17). Earl et al. also performed a competitive assay for anti-gp41 MAbs in ELISA. They also showed that the majority of the anti-gp41 MAbs are conformation dependent and most of determinant I as cluster I MAbs do not compete with determinant II as cluster II MAbs (3). MAb 50.69 was classified as a cluster I antibody, and MAb 98.6 was classified as a cluster II antibody. However, in the present study we performed competitive assays using an infected live cell line and FACS. The difference in the method used may explain the conflicting results. The epitopes of MAbs 50.69 and 98.6, which are expressed on the infected cell surface, possibly have a different conformation from the gp41 peptides previously used in ELISA, while it is known that HIV-1 envelope proteins form oligomers dominantly in viral lysate (6). The envelope proteins on the infected cell surface are heterogeneous with native gp120-gp41 complex, residual gp41 after gp120 shedding, and uncleaved envelope glycoprotein 160 (gp160) precursor, although gp160 derived from viral lysate forms a trimer (10). Atomic force microscopy investigation revealed that monomeric gp120 is dominant on H9/IIIB (8). The unidirectional competition of MAbs 50.69 and 98.6 may be explained by the positional relationship among epitopes on

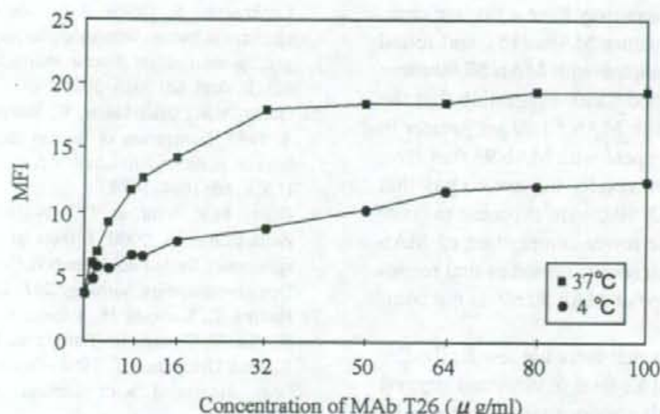

Fig. 1. The temperature dependency of MAb T26 binding. MAb T26 binding to H9/IIIB at 37 C was higher than at 4 C.

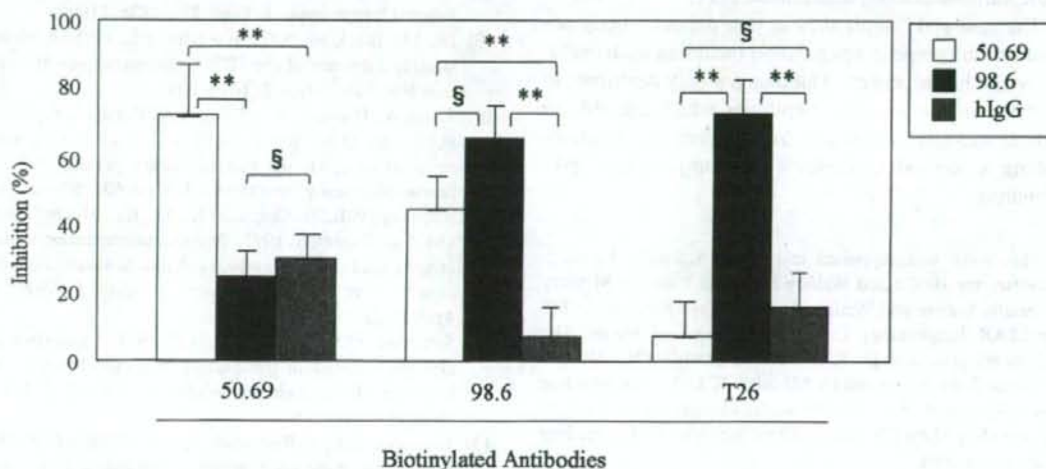

Fig. 2. The decreased binding of biotinylated MAb 50.69, 98.6, or T26 to H9/IIIB cells after preincubation with MAb 50.69 (open bars), 98.6 (filled bars), or hIgG (gray bars). The negative control was calculated as the MFI without preincubation MAb and with biotinylated MAb. The positive control was calculated as the MFI with biotinylated MAb blocked by the same preincubation MAb, the concentration of which was saturated. % inhibition was calculated as [1−(biotinylated MAb MFI−positive control MFI)/(negative control MFI−positive control MFI)]×100 (%). Data are expressed as mean±S.D. Each bar (±S.D.) represents the mean of triplicate determinations (**$P<0.0167$) (§$P \geqq 0.05$). To determine statistically significant differences among the three groups, differences were considered to be statistically significant when $P<0.0167$ by the Bonferroni/Dunn test. Data were analyzed using CellQuest software (Becton Dickinson Biosciences).

monomeric gp41. The size of the epitopes, the induced conformational changes upon binding MAb 50.69, and differences in the affinities of the MAbs also might be responsible for our results.

In this study, we analyzed the binding properties of anti-core structure gp41 human MAbs using infected cells. These analyses will contribute to understanding the structure of gp120-gp41 on the infected cell surface and the complex interactions of humoral antibodies against HIV-1. The unidirectional competition, which has not been able to be observed using gp41-derived peptides so far, suggests that the immune-response against the gp41 core structure varies much among patients and some of them are possibly dominant for certain exclusive epitopes, because patient-derived MAbs are considered to recognize the functional gp41 in the human body. We previously did competition assays between patients' sera and anti-core structure

MAbs, because patients' sera may have a diverse competition with anti-core structure MAbs (15), and found that the patients' sera competed with MAb 50.69 more than MAb 98.6 (unpublished data), suggesting that the antibodies that compete with MAb 50.69 are greater in number than those that compete with MAb 98.6 *in vivo*.

According to the above results the antibodies that bind to the epitope of MAb 50.69 are expected to compete with MAb 98.6. The lower competition of MAb 98.6 with sera suggests that some antibodies that recognize the overlapping epitope of MAb 50.69 do not compete with MAb 98.6.

Our results also indicate that there are few antibodies that have the epitope of MAb 98.6 *in vivo*, and suggest that the antibodies with this epitope may hardly ever be induced, especially among cluster II antibodies. This is assumed to be partly due to the fact that cluster II is a more variable domain than cluster I (11).

The anti-gp41 antibodies in one patient's body are assumed to compose a population including such exclusivity and heterogeneity. Our data not only contribute to analyses of the functional epitopes which are able to induce anti-gp41 antibodies *in vivo*, but also to establishing a method to evaluate the complex anti-gp41 immunity.

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Original Article

YMDD Mutations and Genotypes of Hepatitis B Virus in Northern China

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SUMMARY: The objective of this research was to determine the relationship between YMDD mutations and the genotypes of hepatitis B virus (HBV) during lamivudine treatment. HBV genotypes were determined by nested PCR with 6 pairs of HBV genotype-specific primers (A to F) in serum specimens from 142 hepatitis B patients receiving lamivudine antiviral therapy. YMDD mutations were detected by fluorescent hybridization bioprobe PCR and melting curve assay (FH-PCR-MC). Among 142 serum specimens, 13 samples were genotype B (9.2%), 125 samples were genotype C (88%), 4 samples were genotype D (2.8%), and 80 YMDD mutations were found. The YMDD mutation rates were 69.2 and 54.4% in genotype B and genotype C, respectively. There was no significant difference in the YMDD mutation rate between genotypes B and C. Nine genotype B sera with YMDD mutations were found, including 2 YIDD mutations and 7 YVDD (M + V) mutations. Sixty-eight genotype C sera with YMDD mutations were found, including 34 mutations I (M + I) and 17 mutations V (M + V). There was a significant difference in the YMDD mutation types between genotypes B and C. Our results suggested that the YMDD mutation rate was 56.3% in patients treated with lamivudine for 2-4 years. YIDD was the main mutation type. The YMDD mutation rate showed no significant difference between HBV types B and C ($P > 0.05$), while the YMDD mutation types showed a significant difference between HBV types B and C in Northern China ($\chi^2 = 4.6$, $P < 0.05$).

INTRODUCTION

Hepatitis B, caused by the hepatitis B virus (HBV), exists throughout the world, and the prevalence is especially high in China. There are approximately 1.2 billion people infected by HBV. Lamivudine is one of the nucleoside medicines used to treat HBV. It can improve the condition of chronic hepatitis B patients in terms of virology, biochemistry and histology. Lamivudine cuts down the HBV DNA levels in patients' sera and can produce a certain proportion of HBeAg seroconversion (1). Lamivudine has been promoted for hepatitis B treatment, but the patient's condition may deteriorate rapidly after long-term use. The main reason for this is the occurrence of YMDD mutations in the HBV polymerase gene (2). HBV has been classified into eight genotypes (A - H) based on genome sequence divergences. The genotypes of HBV have distinct geographical distributions. Hepatitis B genotypes are associated with virus replication, virus variation, disease prognosis and the choice of drugs (3-7). In this study, the relationship between the YMDD mutations of HBV and HBV genotypes during lamivudine treatment is determined.

MATERIALS AND METHODS

Study population: A total of 142 serum specimens from patients with chronic hepatitis without cirrhosis who were hospitalized or were being seen in the clinic service at the Infectious Disease Department of the Second Affiliated Hospital of Harbin Medical University, Harbin, China from 2003 to 2005. All cases were diagnosed according to the Standard of Chinese Virus Hepatitis in 2000, and cases with liver complications caused by other aetiologies or other types of hepatitis virus were excluded. The 142 patients included 112 men and 30 women, and their ages ranged from 18 to 78. DNA levels of HBV in serum from all patients were more than 103 copies/ml. All cases received lamivudine treatment orally, 100 mg everyday for 24-48 months, and did not receive other antiviral therapy during the study. All the patients gave written informed consent. This study was approved by the Harbin Medical University Committee on Clinical Investigation.

Laboratory findings: HBeAg levels in patients sera were measured with enzyme-linked immunosorbent assay (ELISA) using the Diagnostic Kit for Hepatitis B e Antigen (PG Biotechnology, Shenzhen, China). The HBV DNA levels were tested by real-time fluorimetry PCR with TaqMan probe using the Quantitative Hepatitis B Virus PCR Fluorogence Diagnostic Kit (PG Biotechnology).

Nucleic acid extraction: HBV DNA amplification was performed for sequence analysis. HBV DNA was extracted from 100 μl test sera and 100 μl of DNA extract I (PG Biotechnology) were oscillated and mixed well, then centrifuged at 13,000 rpm for 10 min, and the supernatant was discarded. Twenty-five microliters of DNA extract II (PG Biotechnology) were added, oscillated and mixed well, and centrifuged at 2,000 rpm for 10 sec. Then, the mixture was placed in a dry bath for 10 min at 100°C and centrifuged at 13,000 rpm for 10 min, and the supernatant was retained in the stock.

Genotyping of HBV by PCR: HBV genotypes were determined by nested PCR with six pairs of HBV genotype-specific primers (A to F). Vide reference (8).

Detection of YMDD mutation: YMDD was detected by fluorescent hybridization bioprobe PCR and melting curve assay (FH-PCR-MC) using the Quantitative Hepatitis B Virus PCR Fluorogence Diagnostic Kit (PG Biotechnology).

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Table 1. HBV genotype distribution and characteristics of the patients

Genotype	Cases	Positive rate	Men/women	Age (mean ± SD)	HBVDNA (10^6 mean ± SD)	HBeAg/HBeAg	Treatment period (month)
Genotype B	13	9.2%	10/3	37.9 ± 11.0	12.9 ± 21.6	9/4	34.2 ± 6.8
Genotype C	125	88.0%	99/26	38.4 ± 11.9	11.3 ± 24.5	95/30	34.9 ± 6.4
Genotype D	4	2.8%	3/1	38.8 ± 11.1	11.7 ± 19.0	3/1	34.8 ± 5.6

Table 2. YMDD mutation rate and YMDD mutation type in 80 HBV sera

Genotype	n	YIDD	YMDD+YIDD	YVDD	YMDD+YVDD	YIDD+YVDD	YMDD mutation rate
Genotype B	9	2 (22.2)	0	6 (66.7)	1 (11.1)	0	69.2% (9/13)
Genotype C	68	30 (44.1)	4 (5.9)	15 (22.1)	2 (2.9)	17 (25.0)	54.4% (68/125)
Genotype D	3	2 (66.7)	0	0	0	1 (33.3)	75.0% (3/4)
Total	80	34 (42.5)	4 (5.0)	21 (26.2)	3 (3.8)	18 (22.5)	56.3% (80/142)

YMDD mutation type was determined after lamivudine treatment for 2 years, and YMDD mutation type was examined every 6 months.

Statistical analysis: Statistical analyses were performed using Adopt SPSS 10.0 software. The rate comparison was made using the chi-square criterion. A difference with a $P < 0.05$ was considered significant. Student's t test and nonparametric test were used to compare differences between groups, where appropriate.

RESULTS

Analysis of HBV genotypes and clinical data: A simple and precise genotyping system based on nested PCR with 6 pairs was developed for the determination of genotypes of HBV. All 142 samples were genotyped by nested PCR analysis: the majority was genotype C, which accounted for 88%; genotype B accounted for 9.2%; and genotype D accounted for only 2.8%. No other genotypes were detected (genotypes A, E, F). No statistically significant differences were observed in mean age, male-to-female ratio, mean serum DNA levels, HBeAg positive and treatment period with genotype B and with genotype C. Genotype D was not included in the statistical analysis because of the low number of cases (Table 1).

YMDD mutation rate and YMDD mutation type in 80 HBV sera: FH-PCR-MC in the detection of HBV YMDD mutation has high sensitivity and specificity. It is a convenient and rapid, and may be used in YMDD typing. HBV wild type and mutation type were detected in 62 and 80 specimens, respectively by FH-PCR-MC. The YMDD mutation rate was 56.3% in the 142 specimens. Among the 80 YMDD mutations, 38 YIDD (M + I) mutations (47.5%), 24 YVDD (M + V) mutations (30%) and 18 YIDD + YVDD mutations (22.5%) were found. Among the 80 YMDD mutations, the YIDD mutation rate was higher than the YVDD mutation rate. The mutation type was mainly YIDD. The YMDD mutation rates were 69.2 and 54.4% in genotypes B and C, respectively. Although the YMDD mutation rate was higher in patients with genotype B than in those with genotype C, there was no statistically significant difference between genotypes B and C ($P > 0.05$). Genotype D was not included in the statistical analysis because of the low number of cases (Table 2).

Relation between the HBV YMDD mutation type and genotypes B and C: Among the 68 patients with genotype C, 23 (33.8%) patients were of the mixed mutation type. Among the 9 patients with genotype B, 1 (11.1%) patient were of the mixed mutation type. Although the YMDD mixed mutation type rate was higher in patients with genotype C than in those with genotype B, there was no statistically significant difference between genotypes B and C ($P > 0.05$). Among the 36 patients with YIDD (M + I) mutation types, 2 patients were genotype B and 34 patients were genotype C. Among the 24 patients with YVDD (M + V) mutation types, 7 patients were genotype B and 17 patients were genotype C. The YMDD mutation types (YIDD and YVDD) showed significant differences between genotypes B and C ($\chi^2 = 4.6$, $P < 0.05$). Genotype D was not included in the statistical analysis due to the low number of cases (Table 3).

Table 3. Relationship between HBV YMDD mutation type and genotypes B, C

Genotype	n	YIDD (M + I)	YVDD (M + V)
Genotype B	9	2 (22.2)	7 (77.8)
Genotype C	51	34 (66.7)	17 (33.3)

DISCUSSION

Lamivudine has been shown to be a potent and nontoxic inhibitor of HBV replication in chronically infected patients. Long-term lamivudine treatment for chronic HBV infection induces the emergence of lamivudine-resistant HBV YMDD mutant strains. In the case of YMDD variants, the methionine (M) is substituted with either isoleucine (I), designated as YIDD, or valine (V), designated as YVDD (9).

It has been reported that the HBV YMDD mutation rate increases along with the duration of lamivudine therapy. The YMDD mutation rates were 16 - 32%, 47 - 56% and 69 - 75% in the groups of patients administered lamivudine for 1, 2 and 3 years, respectively (10). A study from Asia found that the 1-, 2- and 3-year YMDD mutation rates were 15, 38 and 53% after lamivudine therapy, respectively (11). The present 142 chronic hepatitis B patients received lamivudine for 2 - 4 years. The HBV YMDD mutation rate should be 56.3%, based on the previous reports. The mutation type was mainly YIDD. The presence of the YIDD motif preceded the exclusive presence of the YVDD motif, and we concluded that the YIDD motif could occur as a temporal intermediate (12). It has been reported that the YIDD or YVDD motif alone did not shift to the mixed type (YVDD and YIDD) when they were examined 12 months after the detection of the mutant virus in Japan (13). In our study, the YMDD mutation type was determined every 6 months for 2 years after lamivudine

treatment. Recently we found that 2 patients, who were not included in this study, had shifted to the mixed type (YIDD and YVDD) (unpublished observation), indicating that the shift can occur over a relatively long period. During the past few years, many researchers have investigated the forecasting factors of drug resistance and mutations, but without reaching a conclusion. Some factors related to YMDD mutations could increase the curative effect of lamivudine. Recently, many investigations have concentrated on the time of the YMDD mutation appearance, the mutation type, the YMDD mutation and ALT level, the DNA level, and the rate of HBeAg seroconversion, but no relationship between the mutations of HBV and the HBV genotypes has been reported. The viral genotype that represents the features of natural infection is the result of evolutionary variation of the virus. The HBV genotype has a certain association with the route of virus infection, genetic mutations, the progression of chronic hepatitis B, and the efficacy of antiviral therapies. The genotypes of HBV also have distinct geographical distributions. Genotypes B and C have only been found in individuals who originated from eastern Asia and the Far East, including Taiwan (14). In Japan, the great majority of HBV isolates belong to genotype B or C (15). Genotypes B and C are spread dominantly in China, while genotypes A and D relatively rare. Genotype B is prevalent in South China and genotype C in North China (16). The clinical prognosis after infection with HBV varies according to the genotype. In the previous study, the clearance of HBeAg occurred earlier and more frequently, and the development of cirrhosis was less common in patients with genotype B compared to those with genotype C (15). Genotype C is associated with chronic liver disease and genotype B is mostly associated with acute hepatitis in China (17). The present study analyzed 142 sera of chronic hepatitis B in North China. The results showed that genotype C occupied 88%, genotype B 9.2%, and genotype D 2.8%. Genotype C is predominant in patients with chronic liver disease in North China. A study in Japan reported that among 234 patients with chronic hepatitis B infected with genotype A, B, or C and treated with lamivudine for more than 1 year, the emergence of mutations was not different among genotypes A, B, and C as determined by the Kaplan-Meier method (18). In Japan, it was reported that the emergence rate of lamivudine resistance was independent of the genotype (A, B, or C) after the lamivudine treatment of patients with chronic hepatitis B with various genotypes. In contrast, the emergence rate was significantly higher in the Ba (a stands for Asia) subgroup of HBV than in Bj (j for Japan) subgroup ($P < 0.05$) (19). In the Hong Kong study, there was no difference in the antiviral response and the rate of development of YMDD mutations in 82 patients with chronic hepatitis B with genotypes B and C after 1 year of lamivudine treatment (20). In a report on 87 patients receiving lamivudine in Guiyang, the results showed that, though genotype B was dominant in that region, there was a higher mutation rate of drug resistance among genotype C cases (21). In another report, 135 chronic hepatitis B patients received lamivudine for 1 year, and the YMDD mutation rate in genotype C cases was higher than that in genotype B cases in Guangdong (22). But Guiyang and Guangdong provinces are located in the southern parts of China. In the present study, 9 YMDD mutations were found in genotype B, and 68 YMDD mutations were found in genotype C. The YMDD mutation rates were 69.2 and 54.4% in genotype B and in genotype C, respectively, in North China ($P > 0.05$). It has been reported that the YIDD mutation occurs more frequently in genotype D, while YVDD is more common in genotype A (23). It is suggested that the YMDD mutation type is related to the genotype. But the present study showed that the YVDD mutation occurred more frequently in genotype B and the YIDD mutation in genotype C, and that the YMDD mutation showed significant differences between the HBV genotypes B and C ($\chi^2 = 4.6$, $P < 0.05$). It has been reported that the replication ability of the YVDD mutant strain might be stronger than that of the YIDD mutant strain (24). The mutation types among different genotypes are not identical, because the genotypes are associated with virus replication and virus variation. Because of the small number of cases of genotype D, only HBV genotyps B and C were examined in this study. Large-scale prospective studies of each genotype should be conducted in the future to confirm these findings.

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Method for efficient storage and transportation of sputum specimens for molecular testing of tuberculosis

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SUMMARY

SETTING: The polymerase chain reaction (PCR) is a highly sensitive method for the detection of *Mycobacterium tuberculosis* and is available in most countries, though to a lesser extent in rural areas.

OBJECTIVE: To amplify *M. tuberculosis* DNA sequences of sputum spotted on FTA® cards and compare them with the results of microscopic examination among culture-positive samples.

DESIGN: A total of 102 sputum specimens of TB patients in treatment were spotted on FTA cards and stored at room temperature until DNA analysis. We assessed the IS*6110* region of *M. tuberculosis*. The efficacy of the PCR assay for the direct detection of *M. tuberculosis* was evaluated and compared with the results of cultures (Middlebrook 7H9 broth) and smears of fresh sputum specimens.

RESULTS: We were able to detect 10 fg/μl of mycobacterial DNA even after 6 months in storage. The PCR sensitivity and specificity using the FTA card system were 82% and 96%, while microscopic examination showed 41% and 95%, respectively.

CONCLUSION: The FTA® card system for the storage of bacterial DNA from sputum samples should be considered for the molecular diagnosis of tuberculosis. Samples can easily be obtained from geographically isolated populations and shipped by mail for accurate molecular diagnosis.

KEY WORDS: tuberculosis; sputum; PCR; FTA® card

CURRENT GLOBAL TUBERCULOSIS (TB) control efforts are based on the diagnosis of cases followed by adequate treatment. Difficulties involved in the collection, transport and processing of samples in clinical practice have also been a major issue. An initial error made by microscopic diagnosis will not be known until weeks later, when the clinical signs are more evident (in false negatives). Microscopic examination of sputum acid-fast bacilli (AFB) smear is still the most widely available diagnostic tool for TB. Unfortunately, smear microscopy is neither specific for *Mycobacterium tuberculosis*, nor is it very sensitive. Depending on the number of specimens examined, smear microscopy detects 30–60% of culture-positive TB suspects.[1] In most situations in which TB is diagnosed by AFB microscopy, it should be assumed to be *M. tuberculosis* until proven otherwise.[2] Detection of AFB smear-negative patients, who make up a significant proportion of all TB patients, is even more problematic.[3,4] As *M. tuberculosis* grows very slowly, diagnosis by culture is a long process, requiring 3–8 weeks in solid media and 1–4 weeks in liquid media.[5]

The development of rapid and accurate procedures for the diagnosis of TB has been a long-standing goal for two main reasons—to improve case finding and case management, and to improve disease surveillance.[5] Molecular amplification assays such as polymerase chain reaction (PCR), which can specifically amplify large quantities of DNA from small starting quantities (10–100 mycobacteria/ml), have been shown to be a promising alternative even for developing countries.[6,7] Microscopic examination requires $>10^3$ to 10^4 mycobacteria/ml, and it is necessary for the diagnostic laboratory to either process the specimen shortly after collection or store it at 4°C to inhibit the growth of contaminating micro-organisms. The latter procedure entails additional labour costs for the processing and conservation of specimens, and reductions in sensitivity.[8]

The FTA® card system (Whatman International Ltd, Abingdon, Cambridge, UK) was originally developed for storing blood samples for DNA testing; its matrix binds and lyses cells, resulting in amplifiable DNA being immobilised on the paper sections, which can

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be punched out directly for use. The card's size allows storage of several hundred samples at room temperature. The filter is impregnated with a chaotropic agent that denatures infectious agents, and thus, as the samples no longer represent a biohazard, their storage and transport can be managed without burdensome precautions.

In this study, we set out to investigate the sensitivity and specificity of a PCR system for the detection of *M. tuberculosis* in sputum samples spotted on FTA® cards and to evaluate the potential of using this method to overcome the difficulties of transporting and storing sputum samples during the TB diagnosis process.

INDIVIDUALS, MATERIALS AND METHODS

Patient and clinical specimens

A total of 102 sputum specimens from 35 TB patients at the Japanese Red Cross Sendai Hospital were collected prospectively. The clinical diagnosis of TB was established by patient histories and clinical and radiological findings, as recommended by the American Thoracic Society.[9] All the patients provided written informed consent. The study was approved by the Tohoku University Committee on Clinical Investigation and by the Ethics Committee of the Red Cross Hospital.

Evaluation and application of the sputum onto the FTA® cards

Spontaneously produced sputum was the specimen of choice. No patient was assisted by respiratory therapy technicians nor stimulated with hypertonic saline aerosol to produce acceptable sputum. The gross appearance of the sputum was evaluated according to Miller & Jones' classification: M1 (pure mucus), M2 (little purulent content), P1 (purulent sputum less than one third of the volume), P2 (purulent sputum between one third and two thirds of the volume) and P3 (purulent sputum more than two thirds of the volume).[10] Standard precautions, such as the use of gloves and a mask, were taken when manipulating sputum specimens.

To improve the chance of detecting *M. tuberculosis*, we chose the thicker (purulent) particles of the sputum and applied them directly onto a FTA® card using a foam-tipped applicator (Whatman®, Tokyo, Japan) that was squeezed over an area of 2.5 cm in diameter. The card was then allowed to dry for 1 h at room temperature. Heat was not used during the drying period. The cards were then put into storage desiccant packets (Whatman®, Japan) and stored at room temperature until DNA analysis.

Culture and hybridisation

Equal volumes of N-acetyl-L-cysteine/NaOH (4%) were mixed with the specimens for digestion and decontamination. The resulting mixtures were allowed to stand for 15 min at room temperature, then centrifuged at 3000 × g for 15 min. The sediment was re-suspended in 2 ml of phosphate buffered saline (PBS) and 0.5 ml was inoculated into Middlebrook 7H9 broth (Middlebrook, Becton Dickinson, Cockeysville, MD, USA). DNA was extracted in all the culture-positive samples to identify and confirm *M. tuberculosis* using a DNA-DNA calorimetric microdilution plate hybridisation kit (DDH Mycobacteria; Kyokuto Pharmaceuticals, Tokyo, Japan).

Preparation of isolated DNA from FTA® cards

Four discs of 1.2 mm from the spotted area of the filters were cut out using a sterile hole puncher (Harris Micro punch 1.2 mm, Whatman®, Japan) and placed in 1.5 ml PCR tubes. The punch was cleaned by placing the end of the punch in the flame of a Bunsen burner and by sterilisation with 70% ethanol between cuts from different samples. The discs were washed three times for 5 min with 800 μl FTA® purification reagent (Whatman®, Japan), which removes PCR inhibitors and other potential contaminants to ensure the quality of the DNA for downstream analysis. The discs were also rinsed twice with 800 μl TE buffer (10 mM Tris HCL, 0.1 mM EDTA, pH 8.0) (Promega, Tokyo, Japan) for 5 min each time. The discs were then dried at room temperature for 1 h and used directly as templates in the PCR reaction mix in the same tube.

PCR procedure

The primers were synthesised using an Automated Multiplex Oligonucleotide Synthesizer (Roche Diagnostics, Tokyo, Japan) (Table 1). Two different PCR assays were performed. First, as an internal control for monitoring successful DNA extraction, β-globin was amplified by using primers GH21 and PCO3 to yield a 250-bp product.[11] The total reaction volume was 50 μl and the reaction mixture contained each primer (10 pmol each), 2U taq DNA polymerase (Invitrogen Cat N°10342-020), 5 μl 10x PCR buffer (200 mM Tris pH 8.4, 500 mM KCl), 1 μl 10 mM dNTP mixture (Invitrogen Cat N°18427-013), 1.5 μl 50 mM $MgCl_2$ and 38.1 μl distilled water. PCR was performed in an MJ Research PTC-200 DNA Engine Thermal Cycler (MJ Research, Waltham, MA, USA) using the following amplification conditions: 95°C for 5 min, 35 cycles at 95°C for 30 s, 55°C for 45 s, 72°C for 30 s and one cycle at 72°C for 10 min. Finally, a 123-bp sequence of insertion element IS*6110* was amplified using T4-T5 primers.[12] This insertion sequence is present in different numbers and locations in the genomes of most clinical isolates of *M. tuber-*

Table 1 PCR primers used in this study

Target	Sequencing	bp	Ref.
β-globin GH21-PCO3	5′-GGA-AAA-TAG-ACC-AAT-AGG-CAG-3′ 5′-ACA-CAA-CTG-TGT-TCA-CTA-GC-3′	250	10
M. tuberculosis IS*6110* T4-T5	5′-CCT-GCG-AGC-GTA-GGC-GTC-GG-3′ 5′-CTC-GTC-CAG-CGC-CGC-TTC-GG-3′	123	11

culosis, which makes it a useful probe for diagnostic and epidemiological purposes. The final composition of the PCR mix was the same as in the previous case. The PCR conditions were preheating at 94°C for 5 min, then 40 cycles at 94°C for 2 min, 68°C for 2 min, 72°C for 2 min, and then 72°C for 10 min.

Determination of sensitivity

To determine the sensitivity of *M. tuberculosis* detection, 10-fold serial dilutions (1 ng to 10 fg) of H37Rv DNA (kindly provided by Dr I Sugawara, Research Institute of Tuberculosis, Tokyo, Japan) were performed using T4-T5 primers. The final composition and PCR conditions were the same as for amplifying *IS6110*.

All amplification products were detected on 1.5% agarose gel in 1 × TAE buffer stained with ethidium bromide and visualised by ultraviolet transillumination.

Control procedures

A positive control tube containing 0.1 ng H37Rv DNA and a negative control tube containing no DNA were included with each set of reactions. To evaluate cross-contamination during sampling, we performed control punches using unspotted cards.

Statistical methods

The sensitivity and specificity of each pair of primers for the detection of *M. tuberculosis* were calculated on the basis of the study reference standards, with the liquid culture method taken as a gold standard. In addition, the results of individual PCR were employed for the analysis of smear-positive and -negative samples according to the culture results.

RESULTS

Patient characteristics

We evaluated by PCR 102 sputum samples from 35 TB patients (23 male, 12 female), all of whom were receiving anti-tuberculosis treatment for periods ranging from 2 weeks to 1 year. The average age was 51.4 years.

Gold standard

Twenty-two positive culture samples were taken as gold standard. In all of these samples, *M. tuberculosis* was identified by hybridisation assay.

Appearance of the specimens

Eighty-five per cent of smear positives, 68% of culture positives and 81% of PCR positives contained >30% of purulent sputum (P2 or P3 in Miller & Jones' classification[10]).

Sensitivity detection

Four small discs of the FTA® card system were used as templates for the PCR processing. The criterion for using four discs was based on the assumption that the mycobacteria were scanty and heterogeneously distributed during the absorption process; in addition, in a pilot study we determined that using four disks increased the degree of amplification (data not shown). The detection limit of the PCR assay for the amplification of *IS6110* was 10 fg/μl of purified *M. tuberculosis* H37Rv (Figure 1A). The amplification of the 123 bp fragments by PCR using the FTA® card system is depicted in Figure 1C.

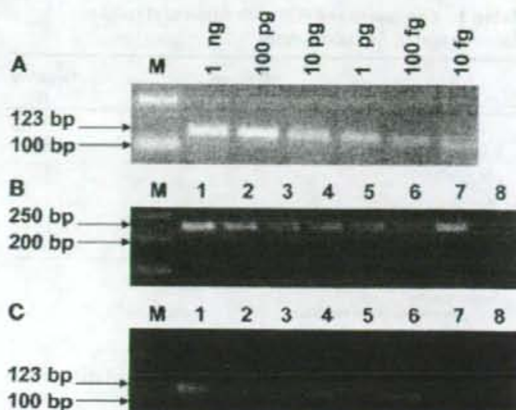

Figure 1 PCR results. **A.** Sensitivity of detection of *M. tuberculosis*: ten-fold serial dilution of H37Rv DNA was amplified. **B.** PCR amplicons from β-globin (250 bp): M: marker; lanes 1–7: results using FTA® cards from TB patients; lane 8: negative control. **C.** PCR amplicons from *M. tuberculosis* (123 bp) stored on FTA® cards: M: marker; lane 1: positive control (H37Rv); lanes 2, 4 and 6: cards from TB patients; lanes 3, 5 and 7: no spotted cards (control punch); lane 8: negative control. In each case, 10 μl samples were electrophoresed through a 1.5% agarose gel and photographed under UV illumination. PCR = polymerase chain reaction; bp = base pairs; TB = tuberculosis; UV = ultraviolet.

Effect of PCR inhibitors

The ability to detect *M. tuberculosis* by PCR can be impaired by the presence of substances inhibitory to Taq DNA polymerase. The β-globin PCR assay generated the expected 250-bp band (Figure 1B) in 90 (89%) of the samples. All the culture-positive samples were also positive in the amplification of β-globin. This finding may suggest that the PCR-negative, culture-positive samples contained low concentrations of TB bacteria rather than PCR inhibitors, which would tend to rule out inhibition as a cause.

PCR and smear results compared with culture

Among the 22 culture-positive samples, 18 (82%) were PCR-positive and 9 (41%) smear-positive (Table 2). The remaining four culture-positive samples were both PCR- and smear-negative. All 9 smear- and culture-positive samples were also positive by PCR. The sensitivity and specificity of PCR were 82% and 96%, compared to 41% and 95%, respectively, for smear examination. Differences were observed on comparing the sensitivity of smear microscopy with that of

Table 2 Comparison of PCR with smear and culture for detection of *M. tuberculosis*

Culture	Positive *n* (%)	Negative *n*
PCR*		
Positive	18 (82)†	3
Negative	4	77
Smear		
Positive	9 (41)†	4
Negative	13	76

* PCR using the FTA® card system.
† (%) sensitivity.
PCR = polymerase chain reaction.

PCR ($P < 0.05$), but there was no observed difference in specificity (Figure 2).

We repeated the PCR procedure for all culture- and smear-positive samples. The second experiment showed the same results as the first.

Sensitivity of PCR by smear result

The sensitivity of PCR for smear-positive, culture-positive samples was 9/9 (100%), whereas that for smear-negative, culture-positive samples was 9/13 (69%). These results show that, even in the paucibacillary form of TB resulting from treatment, this PCR system could provide rapid and sensitive detection of *M. tuberculosis* DNA impregnated on the FTA® card.

Stability and control procedures

All of the 102 samples were analysed by PCR at two time points—at the time of sample collection and after 6 months of storage—obtaining positive amplifications in both cases, clearly showing that storage for 6 months did not affect the amplification. PCR products were not detected in negative controls or control punch cards, confirming the absence of contamination during the procedure (Figure 1C). The total assay time was 9 h.

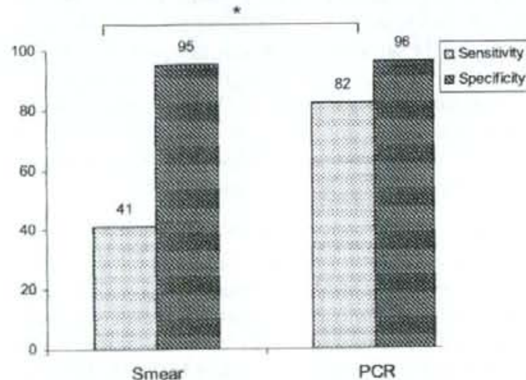

Figure 2 Accuracy of the methods. Sputum smear examination (smear) and PCR assay using the FTA® card system (PCR) among the 22 positive culture samples. * $P < 0.05$. PCR = polymerase chain reaction.

DISCUSSION

The present study demonstrated that the DNA of *M. tuberculosis* can be amplified using sputum spotted on an FTA® card. We found that the *M. tuberculosis* DNA stability with this card at room temperature was up to 6 months. However, care must be taken to avoid cross-contamination between specimens during sampling and handling. The present findings are relevant to patient care and clinical trials and suggest that sputum stored on FTA® cards could provide a simple, economical method for the collection, storage and transport of suspected TB specimens for later testing.

In addition, samples can easily be obtained from geographically isolated populations where access to and/or availability of TB diagnostic testing may be limited. Samples collected may be shipped by mail to a central laboratory for molecular diagnosis without the triple packing system otherwise required for transport.[13]

Depending on the gold standard and other methodological factors, studies have shown PCR sensitivities ranging from 77% to >95% and PCR specificities of >95% in TB patients before treatment.[4,5] Regarding patients under treatment, Kennedy et al. found 76% agreement between culture and PCR.[14] In our study, three culture-negative samples (one smear-positive and two smear-negative) were detected by our method, which may be explained by the limited quantity of TB DNA.[15] While the sensitivity of smear is dependent on the type and quality of the specimen, our method could be employed with accuracy even 6 months after obtaining the sample. However, as we recommend taking the purulent part of the sputum, it will be necessary to help patients understand that secretions from deep in the lung are required.

More sensitive methods exist, including the use of automated culture systems, but the best tests are not always available for the people who most need them.[16]

In summary, the present system appears to be a promising method for transporting and storing sputum samples. Other advantages are the simplicity of the sample preparation and the use of the small disc as a template during the PCR process, using specific targets, without the need for extensive nucleic acid purification.

Acknowledgements

The authors thank Drs J C Agapito and M Rabie for excellent technical assistance and B Bell for reading the manuscript. They also appreciate Dr P Escalante's thoughtful review of and comments on the manuscript.

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RÉSUMÉ

CADRE : La réaction polymérase en chaîne (PCR) est sensible pour la détection de *Mycobacterium tuberculosis*, et est disponible dans la plupart des pays, mais dans une moindre mesure dans les zones rurales.

OBJECTIFS : Amplifier les séquences d'ADN de *M. tuberculosis* des crachats repérées sur les cartes FTA et les comparer avec les résultats des examens microscopiques dans les échantillons de cultures positives.

MÉTHODES : Au total, 102 échantillons de crachats de patients tuberculeux ont été marqués sur les cartes FTA et stockés à l'air ambiant. La spécificité et la sensibilité de deux amorces de PCR qui amplifient la région IS*6110* de *M. tuberculosis* ont été évaluées et comparées à celles de cultures (milieu liquide 7H9), et de l'examen microscopique d'échantillons frais de crachats.

RÉSULTATS : Il a été possible de détecter 10 fg/µl de DNA mycobactérienne même après 6 mois de stockage. L'analyse PCR des deux paires d'amorces révèle une sensibilité et une spécificité respectivement de 82% et 96%, alors que celles de l'examen microscopique sont respectivement de 41% et de 95%.

CONCLUSION : Le système de carte FTA permettant le stockage de l'ADN bactérien issu d'un échantillon de crachats devrait être envisagé pour le diagnostic moléculaire de la tuberculose. Les échantillons de crachats peuvent être facilement obtenus dans des populations géographiquement isolées, stockés et adressés par courrier afin d'établir à distance un diagnostic moléculaire précis.

RESUMEN

CONTEXTO : La reacción en cadena de la polimerasa (PCR) es un método sensible para la detección de *Mycobacterium tuberculosis* y se encuentra al alcance en la mayoría de los países, aunque en menor medida en zonas rurales.

OBJETIVO : Amplificar secuencias del ADN de *M. tuberculosis* a partir de manchas de esputo en papel de filtro (*FTA cards®*) y comparar estos resultados con los resultados de la baciloscopia, en muestras con cultivo positivo para micobacterias.

MÉTODOS : Se recogió un total de 102 muestras de esputo de pacientes con tuberculosis en curso de tratamiento, las cuales se almacenaron como manchas en papel de filtro a temperatura ambiente hasta el momento del análisis. Con la PCR se amplificó un fragmento de 123 pares de bases de la secuencia de inserción IS*6110* de *M. tuberculosis*. Se evaluó la eficacia de la PCR en la detección de *M. tuberculosis* y los resultados se compararon con los resultados de los cultivos en medio líquido 7H9 (método de referencia) y de la baciloscopia, de muestras frescas de esputo.

RESULTADOS : El método permitió detectar hasta 10 fg/µl de ADN micobacteriano en muestras almacenadas durante más de 6 meses. La PCR a partir de las manchas de esputo en tarjetas FTA® mostró una sensibilidad del 82% y una especificidad del 96%, comparada con una sensibilidad del 41% y una especificidad del 95% de la baciloscopia.

CONCLUSIÓN : El sistema con tarjetas FTA® debería tenerse en cuenta como método de conservación del ADN micobacteriano presente en las muestras de esputo, para el diagnóstico molecular de la tuberculosis. Así, en poblaciones geográficamente aisladas, de manera sencilla podrían obtenerse las muestras de esputo, almacenarlas y expedirlas por correo con el fin de establecer un diagnóstico molecular exacto.

Tohoku J. Exp. Med., 2006, **208**, 349-354

Case Report

Serum Antibody Against Granulocyte/Macrophage Colony-Stimulating Factor and KL-6 in Idiopathic Pulmonary Alveolar Proteinosis

Masayuki Nara, Kunio Sano, Hiromasa Ogawa, Tsutomu Tamada, Miyuki Nagaoka, Katsunori Okada,[1] Mika Watanabe,[2] Takuya Moriya,[2] Hiroshi Miki,[3] Koh Nakata,[4] Masakazu Ichinose[5] and Toshio Hattori

Department of Infectious and Respiratory Diseases, [1]Department of Respiratory Surgery, [2]Department of Pathology, Tohoku University School of Medicine, Sendai, Japan, [3]Sendai Medical Center, Sendai, Japan, [4]Bioscience Medical Research Center, Niigata University, Niigata, Japan, [5]The Third Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan

Nara, M., Sano, K., Ogawa, H., Tamada, T., Nagaoka, M., Okada, K., Watanabe, M., Moriya, T., Miki, H., Nakata, K., Ichinose, M. and Hattori, T. *Serum Antibody Against Granulocyte/Macrophage Colony-Stimulating Factor and KL-6 in Idiopathic Pulmonary Alveolar Proteinosis.* Tohoku J. Exp. Med., 2006, **208** (4), 349-354 —— Here we describe a case of idiopathic pulmonary alveolar proteinosis (I-PAP), in which anti-granulocyte/macrophage colony-stimulating factor (GM-CSF) antibody and high level of KL-6 were found in the serum. Anti-GM-CSF antibody is responsible for I-PAP, and KL-6 is a serum marker for the activity of diffuse interstitial lung disease. A 38-year-old woman, who had no symptoms, was found to have an abnormal shadow on chest radiograph 5 years previously at a health check up. Chest radiograph showed a patchy shadow in the left lower lung field. Thoracoscopic biopsy was performed because the shadow had gradually expanded during the 5 years. Histological examination revealed proteinous material filling the alveoli and positive staining by the PAS method, suggesting PAP. Anti-GM-CSF antibody and a high level of KL-6 were detected in the serum at the time of diagnosis. Three years later, the shadow disappeared spontaneously. During this period, the level of KL-6 dramatically decreased, although that of GM-CSF antibody remained unchanged. The present case suggests that the serum level of the anti-GM-CSF antibody represents a useful marker for the diagnosis but not for follow-up of the clinical course. On the contrary, KL-6 is an excellent marker for the assessment of the clinical course of I-PAP. ——
GM-CSF; proteinous material; autoimmune disease; thoracoscopic biopsy
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Pulmonary alveolar proteinosis (PAP) is a rare and enigmatic disorder that is characterized by proteinaceous material accumulation within the alveoli and a variable natural history (Seymour and Presneill 2002; Trapnell et al. 2003). The disease is a heterogeneous group consisting of congenital (C)-PAP and acquired PAP (Seymour and Presneill 2002; Trapnell et al. 2003). More than 90% of the patients with PAP present a primary acquired disorder, that is, idiopathic PAP (I-PAP) (Seymour and Presneill 2002; Trapnell et al. 2003). The primary symptoms in patients with I-PAP are shortness of breath with exercise and/or cough (Wang et al. 1997; Goldstein et al. 1998), and chest radiographs in such patients typically show bilateral air-spaces of a nodular or confluent pattern (Wang et al. 1997). Bronchoalveolar lavage (BAL) and/or lung biopsy (transbronchial [TBLB] or thoracoscopic or open lung biopsy) are needed to confirm the diagnosis (Wang et al. 1997). We describe here the case of a female patient with I-PAP in which 5 years were required to make the diagnosis because of atypical clinical features (e.g., she had no symptoms, her chest radiograph showed a unilateral patchy shadow, routine laboratory data were unremarkable).

Recent investigation has revealed that loss of anti-granulocyte/macrophage colony-stimilating factor (GM-CSF) activity caused by neutralizing anti-GM-CSF autoantibody crippled normal function of alveolar macrophages. This dysfunction that reduces surfactant clearance is responsible of I-PAP (Kitamura et al. 1999; Trapnell et al. 2003). In brief, I-PAP is an autoimmune disease with neutralizing antibody of GM-CSF (Kitamura et al. 1999). In our case, GM-CSF antibody was detected in the serum at the time of diagnosis. And, the level was not changed even after the chest radiograph was improved. On the contrary, the serum level of KL-6 has decreased according to the improvement of the chest radiographic shadow and the diffusing capacity of carbon monoxide (D_{LCO}).

Case Report

A 38-year-old non-smoking woman was found to have an abnormal shadow on chest radiograph at a health check up 5 years prior to visiting our clinic. At that time, when she visited her family doctor, she had no symptoms. The chest radiograph showed a patchy shadow in the left lower lung field without abnormal findings in the peripheral blood, serum and sputum analysis. In the TBLB and BAL no significant findings were obtained. The abnormal shadow gradually spread and new patchy shadows emerged also in the right lung field during the 5 years of observation. On August 5, 2002, she was referred to our clinic for close examination. On admission to our hospital, arterial blood gas values under room air were as follows: PaO_2 82.0 Torr; $PaCO_2$ 40.1 Torr; pH 7.413. Peripheral blood analysis showed normal leukocyte (8,100/μl), red blood cell (4.35 $\times 10^6/\mu$l), platelet ($269 \times 10^3/\mu$l) counts and hemoglobin (12.3 g/dl) level. Also, no laboratory findings suggestive of disorders of the immune system or connective tissue were obtained. C-reactive protein and tumor markers (carcinoembryonic antigen, cytokeratin 19 fragment, progastrin-releasing peptide, and sialyl lewis X antigen) were all negative except for an increase in KL-6 (1,493 U/ml). Her pulmonary function test showed normal function (vital capacity [VC] 2.27L; a prediction of VC 84.4%; forced expiratory volume in one second [FEV_1] 1.82L; a prediction of FEV_1 83.1%) with a reduction in the diffusing capacity for D_{LCO} (D_{LCO} 13.92 ml/min/mmHg; a predictiion of D_{LCO} 70.5%). Sputum examinations were negative for tuberculous bacteria, fungi or malignant cells. Urinalysis was normal. A chest radiograph showed bilateral diffuse infiltrative shadows (Fig. 1A). The patchy shadows were confluent. Chest computerized tomography (CT) scanning showed opacification with a patchy distribution (Fig. 1B). The opacification had a ground-glass appearance. The distribution was widespread but more prominent at the peripheral portion (Fig. 1B). Lung biopsy under video-assisted thoracic surgery (VATS) was performed. In the thoracoscopic survey the left lung showed patches with a change in hue without any effusion and with a smooth or normal surface in both the visceral and parietal pleura. Histological examination of the biopsy specimens from the

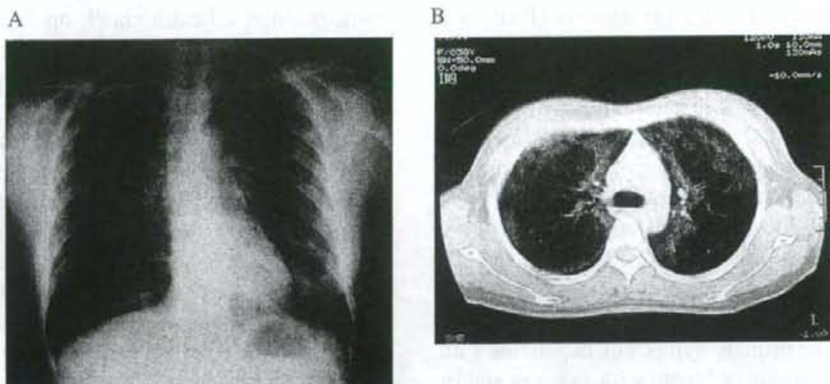

Fig. 1. Radiographic appearance on admission (August 5, 2002). Diffuse infiltrative shadows are seen in both lung fields on chest radiogram (A). Chest computerized tomography (CT) shows opacification with a patchy distribution. The distribution was widespread but more prominent at the peripheral portion (B).

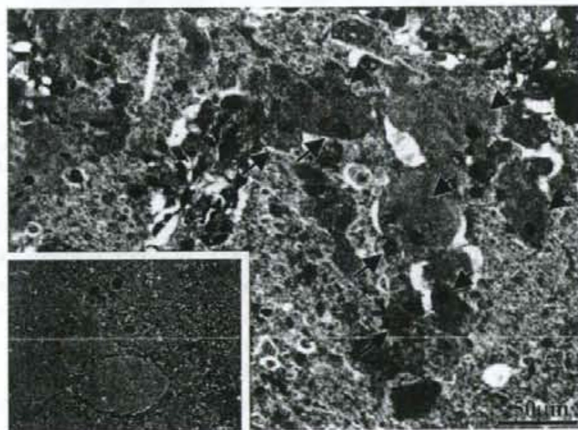

Fig. 2. Light microscopic picture of a lung biopsy specimen under thoracoscopy. Eosinophilic, dense, homogenous material filling in the alveoli can be seen (small magnification, Hematoxyline-Eosin stain). There are collections of foamy histiocytes engulfing the alveolar material (allows in large magnification, Hematoxyline-Eosin stain). Bar: 50 μm.

lingular inferior segment showed eosinophilic dense homogenous material filling in the alveoli (Fig. 2). This precipitate had a fine granular appearance (Fig. 2). The eosinophilic material was periodic acid Schiff reaction (PAS)-positive and diastase resistant. There were also collections of foamy histiocytes engulfing this alveolar material (Fig. 2). The inflammatory reaction was absent to slight in the affected alveoli. Based on these findings, we made the diagnosis of PAP. More than 90% of patients with PAP show a primary acquired disorder of unknown etiology, that is, I-PAP (Seymour and Presneill 2002; Trapnell et al. 2003). I-PAP has been reported as an autoimmune disease with neutralizing antibody of GM-CSF (Kitamura et al. 1999). Kitamura et al. (1999) reported that the GM-CSF-neutralizing antibody was found in sera from all I-PAP patients

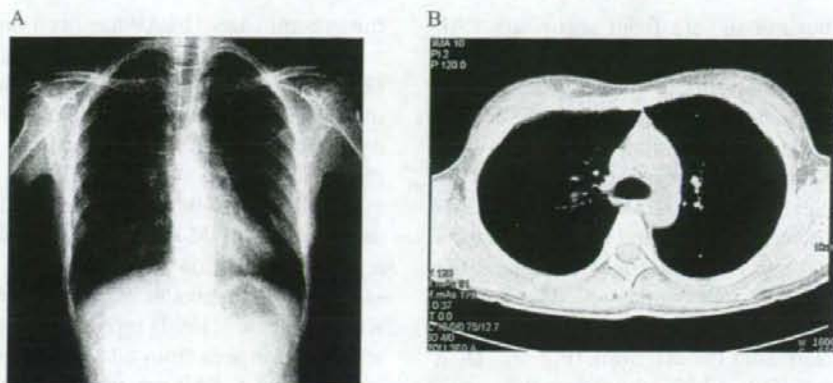

Fig. 3. Radiographic appearance 3 years after the diagnosis (April 25, 2005). Diffuse infiltrative shadows on chest radiogram which were seen on August 5, 2002 had disappeared (A). The opacification with a patchy distribution on chest CT also resolved (B).

Table 1. *Time course of serum anti-GM-CSF antibody, KL-6, pulmonary function data and chest images*

	Aug. 5, 2002	Mar. 29, 2004	Jul. 14, 2005
Serum anti-GM-CSF antibody (μg/ml)	27.66		26.84
KL-6 (U/ml)	1,493	680	156
VC (L)	2.27	2.46	2.54
FEV_1 (L)	1.82	2.13	1.95
D_{LCO} (ml/min/mmHg)	13.92	15.34	20.10
Prediction of D_{LCO} (%)	70.5	84.3	112.3
Chest images	Fig. 1		Fig. 3*

* Chest images in Fig. 3 are taken on April 25, 2005.

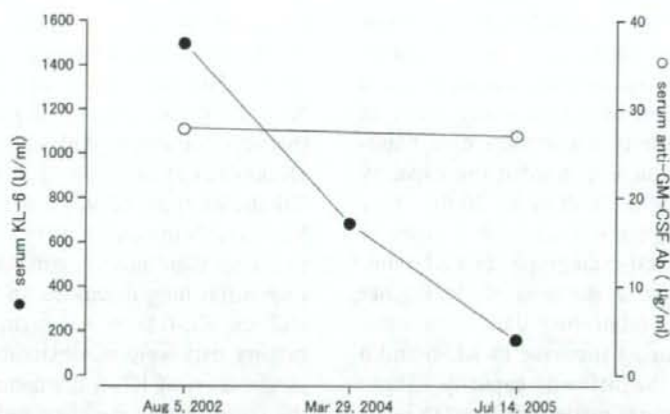

Fig. 4. Time course of serum KL-6 level (●) and anti-GM-CSF antibody (Ab) level (○). The level of KL-6 dramatically decreased, although the level of GM-CSF Ab did not decline.