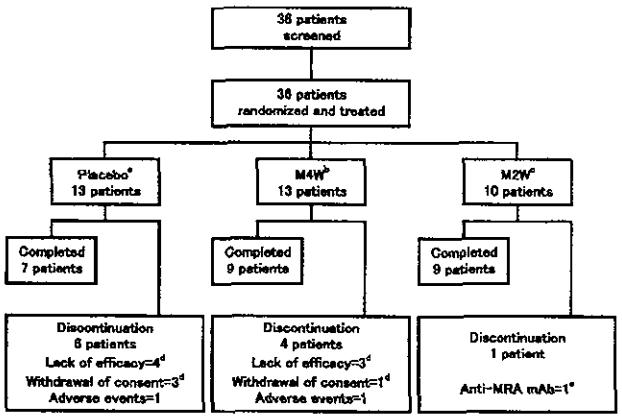

Figure 1. Trial profile of patients with active Crohn's disease who received MRA or placebo by masked randomization. [a]Placebo biweekly; [b]MRA 8 mg/kg/placebo alternately, biweekly; [c]MRA 8 mg/kg biweekly; [d]some patients had more than 1 reason, [e]anti-MRA mAb at baseline was subsequently determined to be a false positive.

nation with the sIL-6R. The captured MRA was detected using a biotinylated monoclonal antibody specific for an epitope in the variable region of MRA, at a dose at the concentration that does not inhibit the binding of IL-6R. The lowest level detected reliably was 1.0 μg/mL.[9]

Statistical Analysis

All patients who received at least 1 dose of the study drug were included in the safety and efficacy assessments. All efficacy comparisons were performed using data from the full analysis set population of patients. The efficacy analysis of an end point was a comparison of the CDAI scores based on the clinical response rates for each MRA regimen with placebo by using the χ^2 test. A P value <0.05 was used to indicate significance.

The χ^2 test was used to test for differences in the remission rates, and the Student t test was used for analyses of parametric data between groups. The final observation for patients discontinued prematurely was carried forward as the final evaluation. All reported P values are 2-sided, and all analyses were performed using SAS (version 8e TS2M0).

Results

Patient Characteristics

Thirty-six patients were randomly assigned to the M2W, M4W, or placebo group (Figure 1). Demographic data for the randomized patients are given in Table 1. There were no significant differences in age, sex, duration of disease, CDAI scores, or laboratory test values including CRP levels at baseline among the groups. All the patients had colonic disease with or without involvement of the small intestine. There were no significant differences in the number of the patients who had undergone previous CD-related surgery among the groups. A similar number of patients in each group had been treated with corticosteroids (4 in placebo, 2.5 to 10 mg/day; 2 in M4W, 5 to 10 mg/day; 2 in M2W, 2.5 to 10 mg/day), mesalamine-derived drugs, metronidazole, and elemental

Table 1. Baseline Characteristics of the Patients

	Placebo[a]	M4W[b]	M2W[c]
Number of patients	13	13	10
Male sex, No. (%)	10 (77)	10 (77)	6 (60)
Age, yr	30.1 ± 7.4	31.0 ± 10.3	32.8 ± 8.2
Weight, kg	53.3 ± 13.7	54.2 ± 5.8	51.2 ± 8.7
Duration of disease, yr	8.6 ± 5.3	7.8 ± 5.7	7.1 ± 5.4
Involved intestinal area, No. (%)			
Small bowel	0	0	0
Small + large bowel	12 (92)	13 (100)	8 (80)
Large Bowel	1 (8)	0	2 (20)
Score of CDAI	294.7 ± 70.2	286.9 ± 65.6	305.7 ± 42.0
CRP, mg/L	31.2 ± 23.2	30.4 ± 22.2	23.4 ± 13.7
Complication, No. (%)	9 (69)	8 (62)	7 (70)
Concurrent infections, No. (%)	0	1 (8)	2 (20)
Previous medications, No. (%)			
Mercaptopurine	0	0	0
Azathioprine	0	1 (8)	0
Corticosteroid	4 (31)	2 (15)	2 (20)
Mesalazine	12 (92)	12 (92)	8 (80)
Salazosulfapyridine	2 (15)	2 (15)	3 (30)
Metronidazole	2 (15)	0	3 (30)
Elemental Diet	10 (77)	10 (77)	8 (80)

NOTE. Plus minus values are means ± SD.
[a]Placebo, biweekly.
[b]MRA, 8 mg/kg/placebo alternately, biweekly.
[c]MRA, 8 mg/kg biweekly.

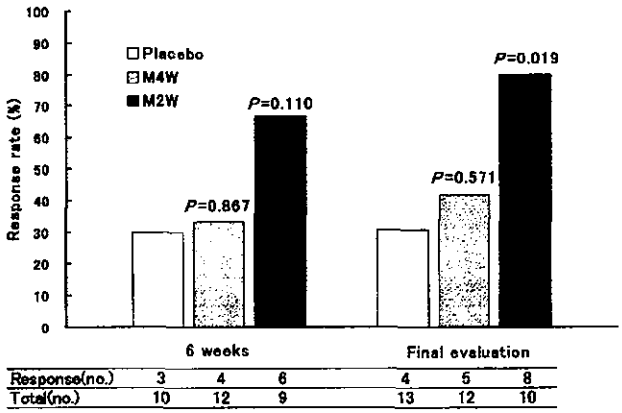

Figure 2. Percentages of patients with Crohn's disease with response (decrease in CDAI score from baseline ≥70 points) according to each treatment group. All significant differences are indicated in the Figure (χ^2 test vs. placebo). *M2W*: MRA 8 mg/kg biweekly; *M4W*: MRA 8 mg/kg/placebo alternately, biweekly; *Placebo*: biweekly.

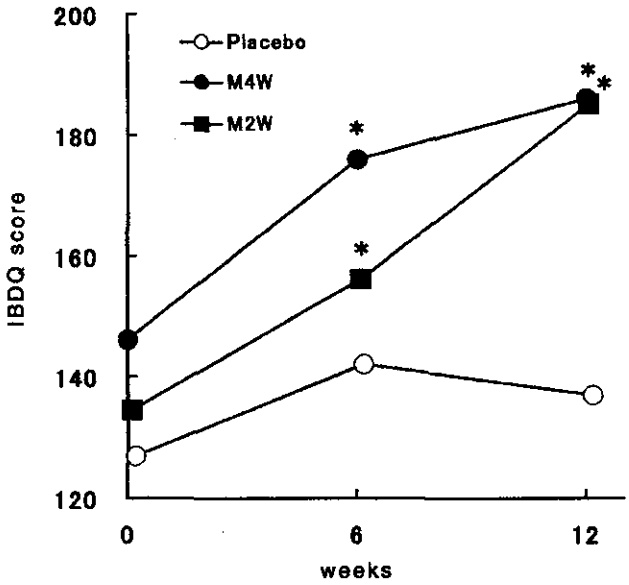

Figure 3. Median IBDQ scores according to each treatment group. *M2W*: MRA 8 mg/kg biweekly; *M4W*: MRA 8 mg/kg/placebo alternately, biweekly; *Placebo*: biweekly. *$P < 0.05$: significantly different from baseline based on paired *t* test.

diet at baseline. Only 1 patient in the M4W group was treated with 60 mg/day of azathioprine; none was treated with mercaptopurine. All treatment groups had a mean CDAI score of approximately 300, despite concomitant medications.

Eleven patients discontinued treatment (6 in placebo, 4 in M4W, and 1 in M2W). The reasons for discontinuation were lack of efficacy or withdrawal of consent (5 in placebo and 3 in M4W) and adverse events (1 each in placebo and M4W). One patient in the M4W group was not assessed for CDAI at 2 weeks because of discontinuation owing to a serious adverse event. One patient in the M2W group was reported positive for anti-MRA antibody at baseline and was discontinued according to the protocol, although it was subsequently determined to be a false positive based on a reassessment of the assay method.

Clinical Response to Treatment

With respect to the primary end point, 80% of the patients in the M2W group had a clinical response at the final evaluation that was statistically significantly higher than 31% of the placebo group (Figure 2). Twenty percent of the patients (2 of 10) on this regimen went into remission, as compared with 0% (0 of 13) of the placebo group ($P = 0.092$). The clinical response rate in the M4W group was 42%. The remission rate in this group (25%; 3 of 12; $P = 0.055$ vs. placebo) was similar to the M2W group.

The mean reduction in the CDAI score in the M2W group was 88 points, from 306 (range, 250–384) to 218 (range, 123–334) points; that in the M4W group was 75 points, from 287 (range, 195–393) to 216 (range, 59–463) points; and that in the placebo group was 41 points, from 295 (range, 183–400) to 254 (range, 164–371).

The quality of life measured by the IBDQ improved in the MRA group (Figure 3). In particular, the MRA groups showed a significant increase in the mean score from baseline at 6 weeks and 12 weeks.

Endoscopic examination was performed in 11 patients and evaluated by using CDEIS. As shown in Table 2, there was no significant difference among the groups. Tissue samples were examined for histology in some patients; however, there was no remarkable improvement either. Patients were on stable doses of corticosteroids

Table 2. Changes in CDEIS From Baseline to Final Evaluation

Group	Patient	Baseline	Final evaluation
Placebo[a]	1	13.6	19.1
	2	13.8	14.0
	3	15.5	24.6
	4	10.6	10.5
M4W[b]	1	19.0	21.7
	2	11.1	20.3
	3	12.2	11.6
	4	5.5	4.6
M2W[c]	1	20.9	18.9
	2	9.7	16.4
	3	19.4	14.6

CDEIS, Crohn's disease endoscopic index of severity.
[a]Placebo, biweekly.
[b]MRA, 8 mg/kg/placebo alternately, biweekly.
[c]MRA, 8 mg/kg biweekly.

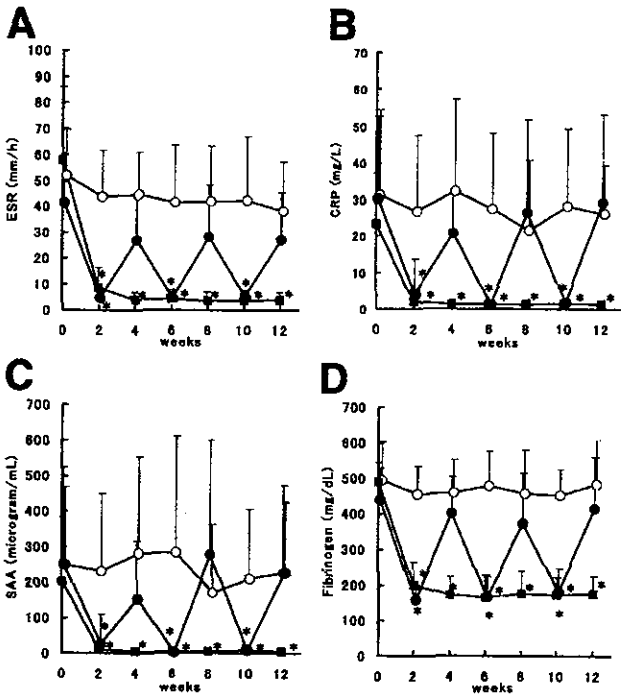

Figure 4. Mean values for (*A*) ESR, (*B*) CRP, (*C*) SAA, and (*D*) fibrinogen concentrations after repeated administration according to each treatment group. ○: Placebo biweekly; ●: MRA 8 mg/kg/placebo alternately, biweekly; ■: MRA 8 mg/kg biweekly. *$P < 0.05$: significantly different from placebo based on Student *t* test. *Bars* indicate SD.

during the study, and steroid-sparing effect was not investigated in this preliminary study.

Inflammatory Markers

The levels of inflammatory markers including ESR, CRP, SAA, and fibrinogen normalized within 2 weeks after a single dose of MRA (Figure 4). The M2W group, but not the M4W group, maintained the normal levels during the trial period. Increased platelet counts seen at baseline decreased to a normal range at 12 weeks: from 375 to 289 $\times 10^3/mm^2$ in the M2W group, from 378 to 378 $\times 10^3/mm^2$ in the placebo group, and from 324 to 313 $\times 10^3/mm^2$ in the M4W group.

Tolerability and Adverse Events

The infusion was generally well tolerated. As presented in Table 3, the adverse events observed in at least 20% of the patients in any of the groups throughout the study were common cold, nausea, pharyngolaryngeal pain, headache, retching, vomiting, and insomnia. Overall, 5 serious adverse events (SAE), which required hospitalization, were reported: 1 in the M2W group, 2 in the M4W group, and 2 in the placebo group. There were 2 SAEs that led to discontinuation from the study. One patient in the M4W group discontinued the treatment because of paralytic ileus, which developed 13 days after the initial infusion. The symptom resolved within 5 days without any intensive treatment. The causal relationship was determined as "possibly" by the investigator. Another patient in the placebo group discontinued treatment because of a suspected intraperitoneal abscess. The remaining 3 SAEs were abdominal pain/gastrointestinal bleeding in the M2W group, gastrointestinal bleeding in the M4W group, and relapse of a perianal abscess in the placebo group. The gastrointestinal bleeding in the M4W group was determined as a "possible" causal relationship. It cannot be explained that the observed paralytic ileus and gastrointestinal bleeding might be related to blockade of IL-6 function. No serious infusion reactions, occurring on any of the infusion days, were reported in any of the treatment groups. No significant trends were observed in the routine laboratory values. No clinically significant abnormalities were found in electrocardiograms, and no deaths occurred during the trial.

Immunologic Results and Pharmacokinetics

No patient developed antinuclear or anti-DNA antibody during the trial period. Specific antibodies to MRA could not be found in the serum from the patients in any of the treatment groups.

Although paired biopsy before and after treatment was performed in only 2 patients, TUNEL-positive, apoptotic mononuclear cells increased in an M2W patient, whereas no remarkable difference was observed in a placebo-treated patient (Figure 5).

The serum concentrations of MRA were detected 2 weeks after every infusion; however, they were no longer detectable values at 4 weeks (Figure 6). Pharmacokinetic analyses revealed that mean half-life of MRA was 113.17

Table 3. Adverse Events

Variable	Placebo[a]	M4W[b]	M2W[c]
Number of patients evaluated	13	13	10
Adverse event, No. (%)			
Common cold	3 (23)	3 (23)	2 (20)
Nausea	2 (15)	3 (23)	2 (20)
Pharyngolaryngeal pain	3 (23)	1 (8)	2 (20)
Headache	2 (15)	1 (8)	2 (20)
Retching	1 (8)	0	3 (30)
Vomiting	1 (8)	1 (8)	2 (20)
Insomnia	1 (8)	0	2 (20)

NOTE. Adverse events that occurred in 20% or more of the patients in any of the groups are reported.
[a]Placebo, biweekly.
[b]MRA, 8 mg/kg/Placebo alternately, biweekly.
[c]MRA 8 mg/kg biweekly.

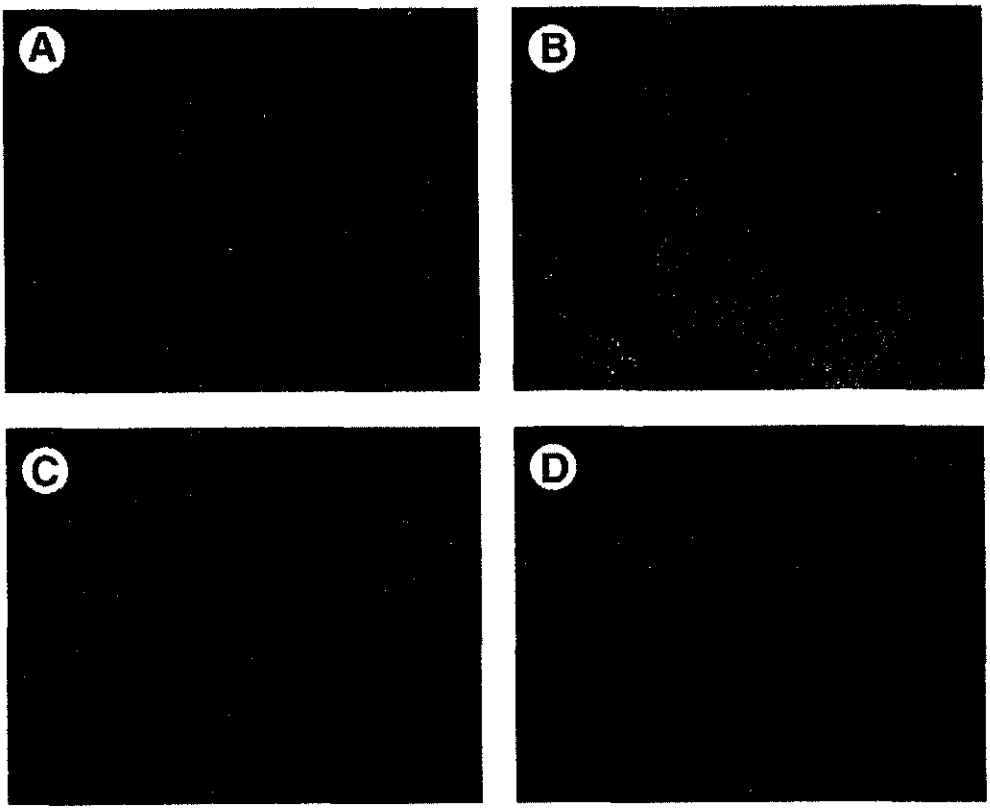

Figure 5. MRA induced apoptosis. Pairs of colonic tissue samples taken from an M2W patient before (*A*) and after treatment (*B*) and from a placebo patient before (*C*) and after treatment (*D*) were stained by using fluorescent TUNEL assay kit (MBL Co., Ltd., Nagoya, Japan; original magnification 100×).

hours in the M2W group and 97.34 hours in the M4W group. The mean volumes of distribution were 63.56 and 64.65 mL/kg in the M2W and M4W groups, respectively. Serum concentration of IL-6 and sIL-6R increased after administration of MRA; however, repetitive infusions of MRA did not induce further increase of these concentrations (Figure 7).

Discussion

Our study is the first randomized placebo-controlled trial of anti-IL-6R mAb MRA in the treatment of patients with active CD. Although this is a preliminary study, the results presented here show that the therapy with MRA for CD is safe and well tolerated and suggests a beneficial effect.

The clinical response rate of the M2W group was higher than that of the M4W group. The different response rate between the MRA groups might be attributable to a continuous suppression of acute-phase reactants such as CRP, ESR, SAA, and fibrinogen for M2W in contrast to M4W, and it is considered that such suppression may require the presence of MRA in the

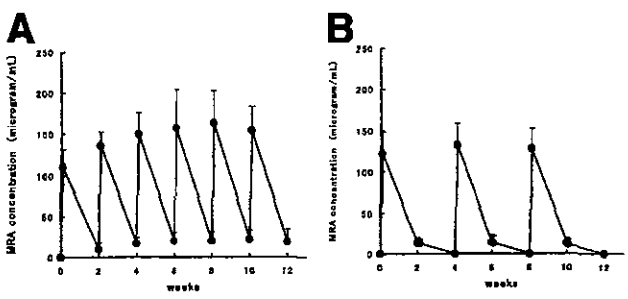

Figure 6. Mean serum MRA concentration (mg/mL) after repeated administration. Serum samples were collected before each infusion and 1 hour after each infusion and at week 12. (*A*) MRA 8 mg/kg were infused biweekly. (*B*) MRA 8 mg/kg or placebo were infused alternately, biweekly. The concentrations were under the limit of detection before each infusion at week 4, 8, and at week 12. *Bars* indicate SD.

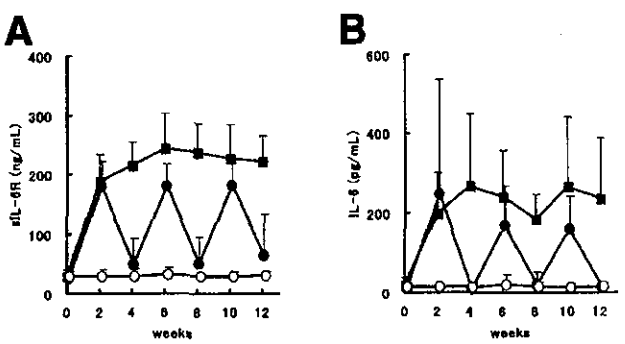

Figure 7. Mean values for (*A*) concentration of IL-6 and (*B*) concentration of sIL-6R after repeated administration according to each treatment group. ○: Placebo biweekly, ●: MRA 8 mg/kg/placebo alternately, biweekly; ■: MRA 8 mg/kg biweekly. *Bars* indicate SD.

serum throughout the treatment period. The levels of acute-phase reactants were completely normalized after only a single dose of MRA. A similar result was observed after administration of MRA for rheumatoid arthritis.[9,13] In studies of other anti-cytokine therapies, such as TNF-α blockers[14–16] and IL-1R antagonist,[17] the acute-phase reactants decreased with the treatment but not to normal levels. Therefore, it is now unquestionable that IL-6 is the principal cytokine responsible for the production of acute-phase reactants in both rheumatoid arthritis and CD. It has been shown that CRP is not a mere serum marker of inflammation but is a promoter of the IL-6R shedding to supply sIL-6R.[18] Therefore, normalization of CRP itself seems to be of benefit in the treatment of CD.

The effect on the anal complications of CD was not included in the principal evaluation of this study; however, 4 out of 6 MRA-treated patients showed disappearance of anal fissure, and 2 out of 10 showed closure of anal fistula, whereas none in the placebo group showed improvement in any of these lesions. Endoscopic and histologic healing was reported after infliximab therapy; however, such healing was not observed in this trial.[19] Although TUNEL staining displayed increased apoptosis of mononuclear cells by MRA treatment, induction of apoptosis was not conclusive because only 1 paired biopsy specimen each from MRA and placebo patient were obtained. Further study is needed to provide definitive results.

It is notable in this pilot study that MRA did not induce autoantibodies or antibodies to MRA itself, although no patients received any immunosuppressive drugs except 1 in the M4W group (azathioprine), in contrast to anti-TNF-α antibody.[14,20] Emergence of autoantibodies such as anti-nuclear antibody and anti-DNA antibody was observed in some patients treated with TNF-α blockers.[20] These antibodies are often seen in patients with systemic lupus, and reduced TNF-α levels were correlated with severe disease in lupus nephritis model,[21] which suggests that TNF-α and IL-6 have different relevance in the autoimmune phenomenon, and blocking the latter might have better safety, although the number of patients was limited in this study. Furthermore, there was no incidence of serious infusion reactions and infections during the treatment period.

This study was performed in Japan, and we have to be circumspect in comparing this study with other therapies because the modalities of the therapy for CD might be different from the Western countries, e.g., the use of steroids, immunosuppressive drugs, and elemental diet. The difference of ethnic background should also be taken into consideration. Therefore, it is desirable that the study of MRA be extended to the countries where other studies were carried out.

In conclusion, this preliminary study shows that a biweekly 8 mg/kg infusion of MRA for 12 weeks is safe and well tolerated by the patients with active CD and showed a significantly higher response rate than placebo, although endoscopic and histologic healing was not observed during the trial period. Striking improvement was observed in the acute-phase reactants, which confirms that IL-6 is the major cytokine responsible for their production in CD. Further work is needed to establish the safety and efficacy of MRA in a larger population of the patients with CD.

References

1. Shanahan F. Crohn's disease. Lancet 2002;359:62–69.
2. Kishimoto T. The biology of interleukin-6. Blood 1989;74:1–10.
3. Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. FASEB J 2001;15:43–58.
4. Mitsuyama K, Toyonaga A, Sasaki E, Ishida O, Ikeda H, Tsuruta O, Harada K, Tateishi H, Nishiyama T, Tanikawa K. Soluble interleukin-6 receptors in inflammatory bowel disease: relation to circulating interleukin-6. Gut 1995;36:45–49.
5. Hosokawa T, Kusugami K, Ina K, Ando T, Shinoda M, Imada A, Ohsuga M, Sakai T, Matsuura T, Ito K, Kaneshiro K. Interleukin-6 and soluble interleukin-6 receptor in the colonic mucosa of inflammatory bowel disease. J Gastroenterol Hepatol 1999;14: 987–996.
6. Yamamoto M, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. J Immunol 2000;164:4878–4882.
7. Atreya R, Mudter J, Finotto S, Müllberg J, Jostock T, Wirtz S, Schütz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schürmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S, Neurath MF. Blockade of interleukin 6 *trans* signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in Crohn disease and experimental colitis in vivo. Nat Med 2000;6:583–588.
8. Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index: National Cooperative Crohn's Disease Study. Gastroenterology 1976;70:439–444.
9. Nishimoto N, Yoshizaki K, Maeda K, Kuritani T, Deguchi H, Sato B, Imai N, Suemura M, Kakehi T, Takagi N, Kishimoto T. Toxicity, pharmacokinetics, and dose-finding study of repetitive treatment with the humanized anti-interleukin 6 receptor antibody MRA in rheumatoid arthritis. Phase I/II clinical study. J Rheumatol 2003; 30:1426–1435.
10. Sato K, Tsuchiya M, Saldanha J, Koishihara Y, Ohsugi Y, Kishimoto T, Bendig MM. Reshaping a human antibody to inhibit the interleukin 6-dependent tumor cell growth. Cancer Res 1993;53: 851–856.
11. Irvine EJ, Feagan B, Rochon J, Archambault A, Fedorak RN, Groll A, Kinnear D, Saibil F, McDonald JW. Quality of life: a valid and reliable measure of therapeutic efficacy in the treatment of inflammatory bowel disease. Gastroenterology 1994;106:287–296.
12. Mary JY, Modigliani R. Development and validation of an endoscopic index of the severity for Crohn's disease: a prospective multicentre study. Gut 1989;30:983–989.

13. Choy EHS, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H, Cheung N, Williams B, Hazleman B, Price R, Yoshizaki K, Nishimoto N, Kishimoto T, Panayi GS. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis. Arthritis Rheum 2002;46: 3143–3150.
14. Targan SR, Hanauer SB, van Deventer SH, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF, Rutgeerts PJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor α for Crohn's disease. N Engl J Med 1997;337:1029–1035.
15. Lipsky PE, van der Heijde DM, St. Clair EW, Furst DE, Breedveld FC, Kalden JR, Smolen JS, Weisman M, Emery P, Feldmann M, Harriman GR, Maini RN. Anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy study group. Infliximab and methotrexate in the treatment of rheumatoid arthritis. N Engl J Med 2000;343:1594–1602.
16. Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, Weaver AL, Keystone EC, Furst DE, Mease PJ, Ruderman EM, Horwitz DA, Arkfeld DG, Garrison L, Burge DJ, Blosch CM, Lange ML, McDonnell ND, Weinblatt ME. Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial. Ann Intern Med 1999;130:478–486.
17. Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, Nuki G, Pavelka K, Rau R, Rozman B, Watt I, Williams B, Aitchison R, McCabe D, Musikic P. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. Arthritis Rheum 1998;41:2196–2204.
18. Jones SA, Novick D, Horiuchi S, Yamamoto N, Szalai AJ, Fuller GM. C-reactive protein: a physiological activator of interleukin 6 receptor shedding. J Exp Med 1999;189:599–604.
19. D'Haens G, Van Deventer S, Van Hogezand R, Chalmers D, Kothe C, Baert F, Braakman T, Schaible T, Geboes K, Rutgeerts P. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: a European multicenter trial. Gastroenterology 1999;116:1029–1034.
20. Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P, and the ACCENT I study group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 2002; 359:1541–1549.
21. Jacob CO, McDevitt HO. Tumour necrosis factor-alpha in murine autoimmune "lupus" nephritis. Nature 1988;331:356–358.

Received April 4, 2003. Accepted January 8, 2004.

Address requests for reprints to: Hiroaki Ito, M.D., Ph.D., Department of Molecular Medicine, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. e-mail: hiroito@imed3.med.osaka-u.ac.jp; fax: (81) 6-6879-3839.

Supported by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

The authors wish to thank Paul Langman, Ph.D., for his valuable assistance with the preparation of the English version of this paper.

The American Journal of Medicine (2005) 118, 181-184

THE AMERICAN JOURNAL of MEDICINE®

BRIEF OBSERVATION

Autoantibodies against carbonic anhydrase II are increased in renal tubular acidosis associated with Sjögren syndrome

Fumi Takemoto, MD,[a] Junichi Hoshino, MD,[a] Naoki Sawa, MD,[a] Yoshifuru Tamura, MD,[b] Tetsuo Tagami, MD,[a] Masafumi Yokota, MD,[a] Hideyuki Katori, MD,[a] Keitaro Yokoyama, MD,[a] Yoshifumi Ubara, MD,[a] Shigeko Hara, MD,[a] Kenmei Takaichi, MD,[a] Akira Yamada, MD,[a] Shunya Uchida, MD[b]

[a]*From the Kidney Center, Toranomon Hospital, Tokyo, Japan; and*
[b]*Department of Internal Medicine, Teikyo University School of Medicine, Tokyo, Japan.*

Sjögren syndrome is an autoimmune disorder characterized by chronic inflammation involving the lacrimal and salivary glands, pancreas, and kidneys.[1] Clinically, renal involvement may manifest as renal tubular acidosis, usually of the distal type,[2,3] perhaps due to tubulointerstitial nephritis.

Serum samples from patients with Sjögren syndrome have several autoantibodies, including anti–carbonic anhydrase II antibody.[4-7] Carbonic anhydrase, which catalyzes the reversible hydration of carbon dioxide to generate a proton and a bicarbonate ion, regulates acid-base homeostasis in erythrocytes, the aqueous chamber of the eye, and the renal tubules.[8] Carbonic anhydrase II, the only type of the enzyme that is soluble, is found in the cytosol of both proximal and distal renal tubular cells.[9] Mice immunized with carbonic anhydrase II develop systemic exocrine gland inflammation that is similar to Sjögren syndrome.[10] We therefore hypothesized that presence of autoantibodies against carbonic anhydrase II may be associated with renal tubular acidosis in patients with Sjögren syndrome.

Requests for reprints should be addressed to Fumi Takemoto, MD, Kidney Center, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan.

E-mail address: ftakemoto-ind@umin.ac.jp.

0002-9343/$ -see front matter
doi:10.1016/j.amjmed.2004.07.049

Methods

We measured levels of anti–carbonic anhydrase II antibody in serum samples from 46 patients who had been diagnosed with primary Sjögren syndrome and who were followed regularly in our outpatient clinic. The diagnosis of Sjögren syndrome required at least four of six standard criteria.[11] No patients had hypocomplementemia or anti-DNA antibodies.

Distal renal tubular acidosis was diagnosed if the arterial bicarbonate level was <22 mEq/L at baseline and the fasting urine pH was >5.5.[12] Ten patients who underwent an oral ammonium chloride loading test[13] were considered to have distal renal tubular acidosis if the minimum urine pH was >5.5. Patients who met either of these criteria were considered to have renal tubular acidosis. Normal controls were selected from age- and sex-matched, disease-free subjects who underwent routine medical examinations at Toranomon Hospital. All subjects gave informed consent; the study was approved by the local ethics committee at Toranomon Hospital.

Serum anti–carbonic anhydrase II antibody levels were measured by an enzyme-linked immunosorbent assay (ELISA) using erythrocyte carbonic anhydrase II (Sigma, St. Louis, Missouri) as a ligand.[6,10] Assays were performed in duplicate; absorbance was determined at 492 nm. We assayed urinary levels of N-acetyl-β-D-glucosaminidase and β_2-microglobulin as markers of renal tubular damages.[14,15]

Table 1 Baseline characteristics of Sjögren syndrome patients and normal controls

Characteristic	Normal controls (n = 19)	Sjögren syndrome	
		Without renal tubular acidosis (n = 33)	Renal tubular acidosis (n = 13)
	Number (%) or Mean ± SD		
Female sex	19 (100)	29 (88)	13 (100)
Age (years)	60.1 ± 8.5	58.2 ± 12.8	61.4 ± 7.4
Arterial bicarbonate (mEq/L)	24.0 ± 1.0	24.2 ± 0.9	21.5 ± 0.8*
Anion gap (mEq/L)	13.3 ± 1.9	13.0 ± 3.0	12.9 ± 3.0
Serum potassium (mEq/L)	4.23 ± 0.28	4.18 ± 0.31	3.65 ± 0.19*†
Serum creatinine (mg/dL)	0.84 ± 0.23	0.71 ± 0.16	1.01 ± 0.67*
Fasting urine pH	5.22 ± 0.56	5.62 ± 0.61†	6.04 ± 0.55*†
Urine NAG (IU/L)	4.6 ± 3.2	4.9 ± 2.5	7.1 ± 6.8
Urine β_2-microglobulin (μg/L)	69 ± 42	136 ± 151	10,058 ± 16,357*†
γ-globulin (%)	16.9 ± 3.0	22.8 ± 6.1†	26.3 ± 5.6*†
IgG (mg/dL)	NA	2023 ± 559	2690 ± 755*
Anti-Ro/SSA (U/mL)	NA	38 ± 74	130 ± 161*
Anti-La/SSB (U/mL)	NA	0.2 ± 1.4	4.5 ± 8.8*
Rheumatoid factor (U/mL)	NA	52 ± 96	30 ± 35
Antinuclear antibody (1: dilution)	NA	1149 ± 1792	1157 ± 842
Schirmer test (right) (mm)	NA	7.8 ± 6.0	3.7 ± 2.0*
Schirmer test (left) (mm)	NA	8.0 ± 5.5	3.5 ± 1.7*
Duration of disease (years)	NA	4.9 ± 3.6	11.1 ± 5.4*
Use of corticosteroids	NA	13 (39)	10 (77)*
Dose of prednisolone (mg/d)	NA	2.1 ± 3.1	4.6 ± 3.2*

ELISA = enzyme-linked immunosorbent assay; IgG = immunoglobulin G; NA = not available or not applicable; NAG = N-acetyl-β-D-glucosaminidase.
*$P < 0.05$ vs. without renal tubular acidosis.
†$P < 0.05$ vs. normal controls.

Statistical analysis

Comparisons were tested for statistical significance using the Student t test or the chi-squared test, as appropriate. Simple regression analysis was conducted by a least-squares method, and multiple regression analysis was conducted using forward selection if the F value exceeded 4.0. Parameters that were associated independently with the arterial bicarbonate level (at $P < 0.05$) were entered into a logistic regression model that used renal tubular acidosis as a dichotomous outcome. P values < 0.05 were considered to indicate statistical significance.

Results

Of the 46 patients with primary Sjögren syndrome, 13 were diagnosed with distal renal tubular acidosis, including 7 who underwent the ammonium chloride loading test (Table 1). Patients with distal renal tubular acidosis had lower serum levels of bicarbonate and potassium, and higher urine pH, than did patients in the group without renal tubular acidosis. They also had higher levels of serum creatinine, γ-globulin, immunoglobulin G, anti-Ro/SSA, and anti-La/SSB; a longer duration of disease; previous or current corticosteroid therapy; and poorer results on the Schirmer test. Although urine N-acetyl-β-D-glucosaminidase levels did not vary among the three groups, urine β_2-microglobulin levels were elevated markedly in the renal tubular acidosis group.

Mean (± SD) serum anti–carbonic anhydrase II antibody levels were greater in patients with renal tubular acidosis than in those without (1.46 ± 0.42 ELISA unit/mL vs. 0.80 ± 0.22 ELISA unit/mL, $P = 0.001$; Figure 1); both patient groups had greater levels than did normal control subjects (0.53 ± 0.16 ELISA unit/mL). Anti–carbonic anhydrase II antibody levels correlated positively with increased urine β_2-microglobulin levels and disease duration, and correlated inversely with serum potassium and arterial bicarbonate levels (Table 2; Figure 2).

In a multiple linear regression analysis, three parameters correlated with arterial bicarbonate levels: anti–carbonic anhydrase II antibody levels, urine β_2-microglobulin levels, and duration of disease. In a logistic regression model, anti–carbonic anhydrase II antibody levels (odds ratio [OR] = 1.9 per 0.1 ELISA unit/mL increase; 95% confidence interval [CI]: 1.2 to 2.8 per 0.1 ELISA unit/mL increase; $P = 0.02$) and duration of disease (OR = 1.4 per 1-year increase; 95% CI: 1.1 to 1.9 per 1-year increase; $P = 0.02$) were associated with the presence of renal tubular acidosis.

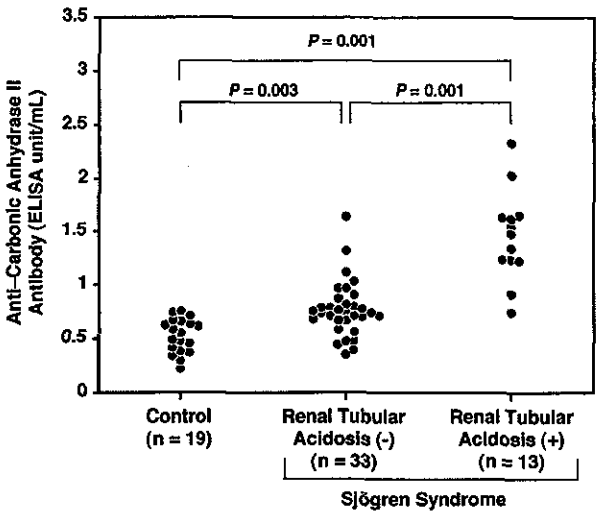

Figure 1 Serum anti-carbonic anhydrase II antibody levels in 19 normal controls and 46 patients with Sjögren syndrome, 13 of whom had distal renal tubular acidosis. ELISA = enzyme-linked immunosorbent assay.

Discussion

We found that among patients with Sjögren syndrome, those with distal renal tubular acidosis had higher levels of anti-carbonic anhydrase II antibody than did those without renal tubular acidosis, although there was some overlap in antibody levels. Although our results cannot establish a causal relation, disordered function of carbonic anhydrase II may be involved, since patients with congenital carbonic anhydrase II deficiency have presented with renal tubular acidosis.[16,17]

It is not known how autoantibodies against carbonic anhydrase II arise, or how these antibodies affect the function of carbonic anhydrase in cortical collecting ducts. Antibody might be produced in response to tubular damage that has exposed intracellular carbonic anhydrase II to the immune system. Inflammation would allow the antibody to enter the cytosolic compartment and inhibit the catalytic activity of the enzyme, as suggested by an in vitro study.[18] Alternatively, carbonic anhydrase II antibodies might just be markers of damage to tubular cells. Indeed, patients with renal tubular acidosis also had elevated β_2-microglobulin levels in urine, consistent with tubular damage. One previous study reported that serum β_2-microglobulin levels were slightly higher in patients with Sjögren syndrome who had inadequate urinary acidification.[19] Serum β_2-microglobulin levels in that study were also related to duration of xerostomia, duration of disease, and serum creatinine levels, perhaps indicating more extensive lymphocyte infiltration and thus greater severity of disease.[19] We did not measure serum β_2-microglobulin levels; the marked elevations in urinary β_2-microglobulin levels likely reflect tubulointerstitial lesions.

In conclusion, our results raise the possibility that distal renal tubular acidosis in Sjögren syndrome may be caused, at least in part, by defective function of carbonic anhydrase II resulting from high plasma levels of carbonic anhydrase II autoantibodies. Tubulointerstitial lesions resulting in an increase in urine β_2-microglobulin levels may underlie the pathogenesis of the disorder. Future studies should determine whether the serologic and urinary abnormalities precede—or are a consequence of—the development of renal pathology in patients with Sjögren syndrome.

Table 2 Correlations between various parameters and serum anti-carbonic anhydrase II antibody and arterial bicarbonate levels in Sjögren syndrome (n = 46)

Correlation of:	With:	Correlation coefficient (*r*)	*P* value
Anti-carbonic anhydrase II antibody	Serum potassium	−0.55	0.001
	Serum creatinine	0.13	0.001
	Arterial bicarbonate	−0.57	0.001
	Serum γ-globulin	0.17	0.27
	Serum anti-Ro/SSA	0.24	0.11
	Serum anti-La/SSB	0.27	0.07
	Urine NAG	0.26	0.08
	Log (urine β_2-microglobulin)	0.38	0.01
	Duration of disease	0.35	0.02
	Schirmer test	−0.23	0.13
Arterial bicarbonate	Serum potassium	0.56	0.001
	Serum creatinine	0.52	0.001
	Serum γ-globulin	−0.27	0.07
	Serum anti-Ro	−0.38	0.009
	Serum anti-La	−0.30	0.04
	Urine NAG	−0.16	0.29
	Log (urine β_2-microglobulin)	−0.53	0.001
	Duration of disease	−0.45	0.001
	Schirmer test	0.45	0.002

NAG = N-acetyl-β-D-glucosaminidase.

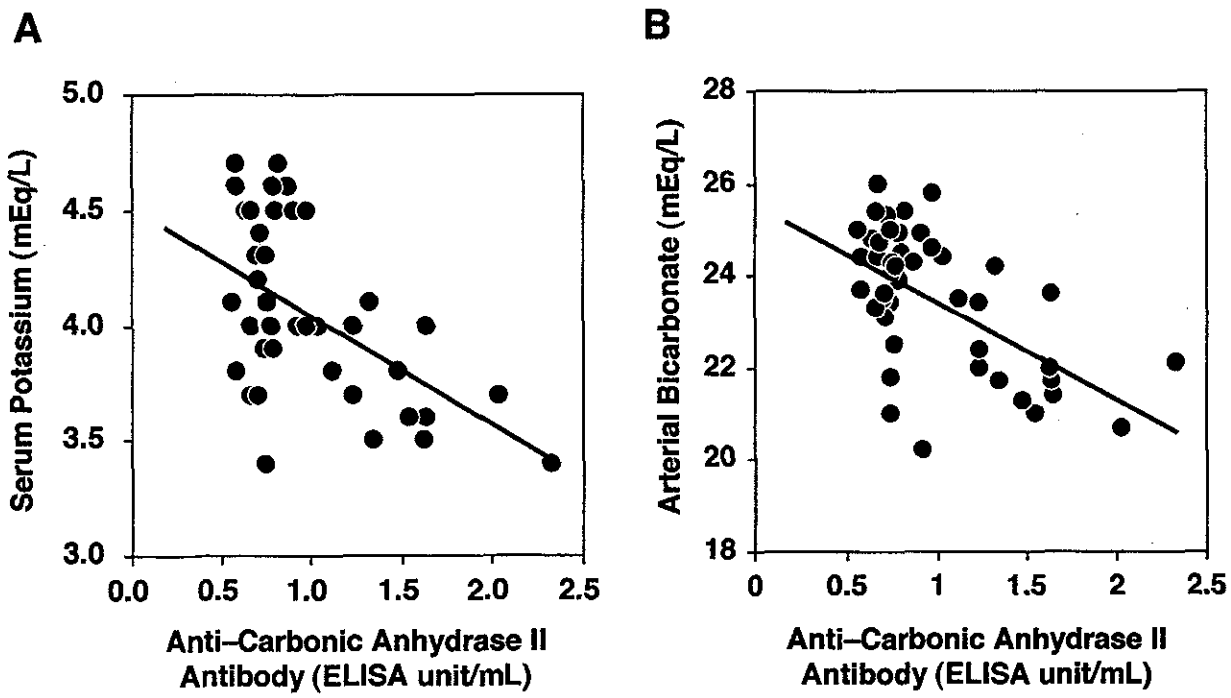

Figure 2 Correlation of serum anti–carbonic anhydrase II antibody levels with serum potassium and arterial bicarbonate levels. (A) serum potassium ($r = -0.55$, $P = 0.001$). (B) arterial bicarbonate ($r = -0.57$, $P = 0.001$). ELISA = enzyme-linked immunosorbent assay.

Acknowledgment

We are grateful to Drs. Hiroshi Kanbayashi and Yuzo Endo for their useful suggestions.

References

1. Fox RI, Howell FV, Bone RC, Michelson P. Primary Sjögren syndrome: clinical and immunopathologic features. *Semin Arthritis Rheum.* 1984;14:77–105.
2. Shioji R, Furuyama T, Onodera S, Saito H, Ito H, Sasaki Y. Sjögren's syndrome and renal tubular acidosis. *Am J Med.* 1970;48:456–463.
3. Talal N. Sjögren's syndrome, lymphoproliferation, and renal tubular acidosis. *Ann Intern Med.* 1971;74:633–634.
4. Inagaki Y, Jinno-Yoshida Y, Hamasaki Y, Ueki H. A novel autoantibody reactive with carbonic anhydrase in sera from patients with systemic lupus erythematosus and Sjögren's syndrome. *J Dermatol Sci.* 1991;2:147–154.
5. Itoh Y, Reichlin M. Antibodies to carbonic anhydrase in systemic lupus erythematosus and other rheumatic diseases. *Arthritis Rheum.* 1992;35:73–82.
6. Kino-Ohsaki J, Nishimori I, Morita M, et al. Serum antibodies to carbonic anhydrase I and II in patients with idiopathic chronic pancreatitis and Sjögren's syndrome. *Gastroenterology.* 1996;110:1579–1586.
7. Fox RI, Tornwall J, Maruyama T, Stern M. Evolving concepts of diagnosis, pathogenesis, and therapy of Sjögren's syndrome. *Curr Opin Rheumatol.* 1998;10:446–456.
8. Maren TH. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol Rev.* 1967;47:595–781.
9. Lonnerholm G, Ridderstrale Y. Intracellular distribution of carbonic anhydrase in the rat kidney. *Kidney Int.* 1980;17:162–174.
10. Nishimori I, Bratanova T, Toshkov I, et al. Induction of experimental autoimmune sialoadenitis by immunization of PL/J mice with carbonic anhydrase II. *J Immunol.* 1995;154:4865–4873.
11. Vitali C, Bombardieri S, Jonsson R, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis.* 2002;61:554–558.
12. Halperin ML, Carlisle EJF, Donnelly S, Kamel KS, Vasuvattakul S. Renal tubular acidosis. In: Narins RG, ed. *Maxwell & Kleeman's Clinical Disorders of Fluid and Electrolyte Metabolism.* 5th ed. New York, New York: McGraw-Hill; 1994:875–910.
13. Wrong O, Davies HE. The excretion of acid in renal disease. *QJM.* 1959;28:259–313.
14. Sherman RL, Drayer DE, Leyland-Jones BR, Reidenberg MM. N-acetyl-β-glucosaminidase and β_2-microglobulin. Their urinary excretion in patients with renal parenchymal disease. *Arch Intern Med.* 1983;143:1183–1185.
15. Statius van Eps LW, Schardijn GH. Value of determination of β_2-microglobulin in toxic nephropathy and interstitial nephritis. *Wien Klin Wochenschr.* 1984;96:673–678.
16. Sly WS, Hewett-Emmett D, Whyte MP, Yu YS, Tashian RE. Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proc Natl Acad Sci U S A.* 1983; 80:2752–2756.
17. Sly WS, Whyte MP, Sundaram V, et al. Carbonic anhydrase II deficiency in 12 families with the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *N Engl J Med.* 1985;313:139–145.
18. Botre F, Botre C, Podesta E, Podda M, Invernizzi P. Effect of anticarbonic anhydrase antibodies on carbonic anhydrases I and II. *Clin Chem.* 2003;49:1221–1223.
19. Pertovaara M, Korpela M, Kouri T, Pasternack A. The occurrence of renal involvement in primary Sjögren's syndrome: a study of 78 patients. *Rheumatology (Oxf).* 1999;38:1113–1120.

Jpn J Clin Oncol 2003;33(9)470–476

Influence of Clinical Parameters on Quality of Life During Chemotherapy in Patients with Advanced Non-small Cell Lung Cancer: Application of a General Linear Model

Satoshi Morita[1], Kunihiko Kobayashi[2], Kenji Eguchi[3], Taketoshi Matsumoto[4], Masahiko Shibuya[5], Yasufumi Yamaji[6], Junichi Sakamoto[1] and Yasuo Ohashi[7]

[1]Department of Epidemiological and Clinical Research Information Management, Kyoto University Graduate School of Medicine, Kyoto, [2]Department of Pulmonary Medicine, Saitama Cancer Center, Saitama, [3]Division of Pulmonary Medicine, Tokai University School of Medicine, Kanagawa, [4]Department of Respiratory Disease, Musashigaoka Hospital, Kumamoto, [5]Division of Respiratory Medicine, Tokyo Metropolitan Komagome Hospital, Tokyo, [6]Department of Internal Medicine, Mitoyo General Hospital, Kagawa and [7]School of Health Sciences and Nursing, University of Tokyo, Tokyo, Japan

Received January 5, 2003; accepted August 12, 2003

Objective: The aim of this study was to determine the relative influence of physician-assessed clinical parameters, including non-hematological adverse events and performance status, on quality of life (QOL) during chemotherapy.
Methods: QOL questionnaires consisting of four domains (functional, physical, mental and psychosocial) were self-administered every week during chemotherapy by patients with advanced non-small cell lung cancer in two phase III clinical trials; 377 patients who completed the questionnaires at baseline and at least once during the first course of therapy were analyzed. A general linear model was applied, where the four domains and the clinical parameters (nausea/vomiting, anorexia, diarrhea, fever, peripheral neuropathy and performance status) were used as the response and explanatory variables, respectively. In this model, the multi-dimensional and longitudinal aspects of QOL data were taken into account.
Results: All four domains were significantly affected by the occurrence of nausea/vomiting, anorexia and diarrhea. No influence of peripheral neuropathy on the domains was detected. Performance status was significantly related to the domains (except the psychosocial domain).
Conclusion: This study revealed, by examination of multi-dimensional repeated QOL data, that clinical parameters had significant effects on QOL in patients undergoing chemotherapy. Our findings suggest that supportive care to control non-hematological adverse events, especially gastrointestinal, could maintain overall QOL in cancer patients in an earlier phase of chemotherapy.

Key words: quality of life – non-small cell lung cancer – chemotherapy – adverse event – general linear model

INTRODUCTION

In the late 1980s and early 1990s, active discussion took place about the definition of quality of life (QOL) and the methodology for assessing QOL in cancer patients (1–7). Today, the consensus view holds that QOL is a subjective, multi-dimensional construct of both positive and negative domains (3,4,6,8), which should, at a minimum, include functional, physical, mental and social well-being (9). These domains are thought to be conceptually related to one another at a moderate correlation (1,2). A number of well-validated cancer-specific instruments for QOL assessment have been developed, such as the European Organization for Research and Treatment of Cancer (EORTC) core questionnaire QLQ-C30 (2) and the Functional Assessment of Cancer Therapy Scale – General (FACT-G) (10). In addition, the QOL Questionnaire for Cancer Patients Treated with Anticancer Drugs (QOL-ACD) was developed by the Japanese Quality of Life Research Group as a generic questionnaire based on a multi-dimensional construct (11). Subsequently, QOL has been investigated in a longitudinal fashion in clinical trials of cancer therapy using those ques-

For reprints and all correspondence: Satoshi Morita, Department of Epidemiological and Clinical Research Information Management, Kyoto University Graduate School of Medicine, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. E-mail: smorita@pbh.med.kyoto-u.ac.jp

tionnaires as an important supplementary endpoint to traditional endpoints such as survival, tumor response and toxicity.

Several studies have investigated the relationship between patient-reported QOL and physician-assessed clinical parameters, such as toxicity and performance status, owing to concern about patients' tolerance of chemotherapy (12–17). These studies, however, were not necessarily adequate to capture the entire effect of clinical parameters on QOL, because of the following factors: (1) they did not analyze longitudinal data, even though a longitudinal analysis is expected to provide added efficiency (statistical power) in estimating the associations between clinical parameters and QOL relative to a cross-sectional analysis (18); (2) they did not examine multi-domain QOL data despite the multi-dimensional aspect of QOL; or (3) they evaluated the effect of one of the clinical parameters on QOL without adjusting for other parameters, thereby preventing an evaluation of their relative effects.

This study investigated multi-dimensional QOL data weekly during chemotherapy using a general linear model, in order to determine the relative influence of physician-assessed clinical parameters on QOL in patients with advanced non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS

This was an ancillary study for two clinical trials (trials A and B), which were conducted by, respectively, the West and East CPT-11 Lung Cancer Study Groups in Japan to compare three chemotherapy regimens using common endpoints and treatment/evaluation schedules. Previously untreated patients with stage IIIB/IV NSCLC and an ECOG performance status (PS) of 0–2 were eligible for these trials. The primary endpoint of these trials was overall survival. Toxicity, response rate and time to disease progression were secondary endpoints and QOL was also investigated in the trials. In trial A (July 1995 to January 1998), 398 patients were randomized to receive one of the following regimens: (1) cisplatin (80 mg/m^2 on day 1) and irinotecan (60 mg/m^2 on days 1, 8 and 15), (2) cisplatin (80 mg/m^2 on day 1) and vindesine (3 mg/m^2 on days 1, 8 and 15) or (3) irinotecan alone (100 mg/m^2 on days 1, 8 and 15), with all schedules repeated every 4 weeks. In trial B (June 1995 to October 1997), 210 patients were randomized to receive either cisplatin and irinotecan or cisplatin plus vindesine by the same schedules as in trial A.

QOL Assessment and Evaluation of Clinical Parameters

In both trials, QOL was measured by the QOL-ACD. This questionnaire has been previously validated for patients with advanced NSCLC (19). The QOL questionnaires were self-administered by patients prior to treatment, every week during treatment (before infusion or just before days 1, 8, 15 and 22 of each course) and for 1 month after the last administration and monthly thereafter. We analyzed the data observed at baseline and in the first course (weeks 1–4) in patients who completed at least two QOL questionnaires (i.e. one before treatment plus one or more during the first course), in order to avoid the possible influence of disease progression on QOL and because we expected that a considerable amount of missing data would occur after the first course.

The QOL-ACD is a 22-item questionnaire covering four domains evaluating functional well-being (items 1–6), physical well-being (items 7–11), mental well-being (items 12–16) and psychosocial well-being (items 17–21). A face scale (item 22) was also used to evaluate global QOL (Fig. 1). The validation study found that two items (item 6 owing to its low test–retest reliability and item 16 owing to its poor convergent validity) should be excluded from QOL evaluation of NSCLC patients, at least in the inpatient setting (19). The score for each domain was obtained by calculating the average of the items (except items 6 and 16) completed by the patient and then applying a linear transformation to obtain an average score ranging from 0 to 100. For all items and domains, higher scores represent better QOL.

Non-hematological adverse events (nausea/vomiting, anorexia, diarrhea, fever and peripheral neuropathy) and the ECOG PS were the clinical parameters used for analysis. The World Health Organization (WHO) criteria (20) were used to grade adverse events, except diarrhea, which was assessed according to the ECOG common toxicity criteria (21). The most severe adverse events and the worst PS experienced within each week were computed for each patient.

Statistical Analysis

Mean QOL scores for the four domains were calculated at baseline and in each week during the first course of chemotherapy. Frequencies of patients who experienced adverse events in each week during the first course were also calculated. In addition, QOL scores obtained during the second course of chemotherapy were analyzed to examine whether the same longitudinal profiles of QOL were observed as during the first course. Regarding the influence of clinical parameters on QOL, multivariate longitudinal data from baseline through the first course were analyzed using a general linear model, with a covariance structure designed to account for correlations both among domains and across time (18,22,23). For estimating the relative effects of the clinical parameters on the four domains, changes in the scores for the four domains in the questionnaire from baseline were used as response variables and the severity of non-hematological adverse events and the changes in PS from baseline were used as explanatory variables.

Additional general linear model analyses stratified by gender (male/female), age (<64 or ≥64 years) and treatment arm (cisplatin + irinotecan/cisplatin + vindesine/irinotecan alone) were performed to examine whether quantitative or qualitative differences existed in the relationship of the clinical parameters with the four domains in such subgroups.

Although some data were expected to be missed even in the first course, all available QOL data from each of the weeks 1–4 were used in these analyses, under the assumption that data missed were missed at random and therefore ignorable (24,25).

QOL-ACD

This questionnaire will help us understand your current condition.

Please circle the number that best describe your condition in the past few days. (This information will remain strictly confidential and will not in any way affect your therapy. Please answer exactly the way you feel.)

1. How much were you able to accomplish your daily activity? 1 2 3 4 5 — Not at all — Fully accomplished
2. How often were you able to go out without help? 1 2 3 4 5 — Not at all — Did not need any help at all
3. Were you able to take a half hour walk? 1 2 3 4 5 — Not at all — Without any problem
4. Did you feel any difficulty walking even a short distance? 5 4 3 2 1 — Did not have any problem — Very much
5. Were you able to walk up and down the stairs? 1 2 3 4 5 — Not at all — Yes, no problem
6. Were you able to take a bath by yourself? 1 2 3 4 5 — Not at all — Yes, without any help
7. How well did you feel? 1 2 3 4 5 — Very poor — Very well
8. Did you have a good appetite? 1 2 3 4 5 — Not at all — Very much
9. Did you enjoy your meals? 1 2 3 4 5 — Not at all — Very much
10. Did you experience any vomiting? 5 4 3 2 1 — None — Very often
11. Did you lose any weight? 5 4 3 2 1 — No, I have rather gained weight — Yes
12. Did you sleep well? 1 2 3 4 5 — Not at all — Very well
13. Were you able to devote yourself on (become enthusiastic about) something? 1 2 3 4 5 — Not at all — Very much
14. How well were you able to deal with your stress? 1 2 3 4 5 — Not at all — Very well
15. *Did you feel you could not concentrate on something?* 5 4 3 2 1 — Yes, very much — Not at all
16. Did you get any encouragement from something/somebody you believe/trust (e.g., family, friends, religion, hobby)? 1 2 3 4 5 — Not at all — Very much
17. Did you worry about your disease? 5 4 3 2 1 — Not at all — Very much
18. Did you have any problem dealing with people outside your family? 5 4 3 2 1 — No problem — Very much
19. Did you think your family was troubled by your getting treatment? 5 4 3 2 1 — Not at all — Very much
20. Do you worry about your social life in the future? 5 4 3 2 1 — Not at all — Very much
21. How much do you worry about your financial problem caused by your treatment? 5 4 3 2 1 — Not at all — Very much
22. Please circle the number of *the face that best fits your feelings in the past few days?*

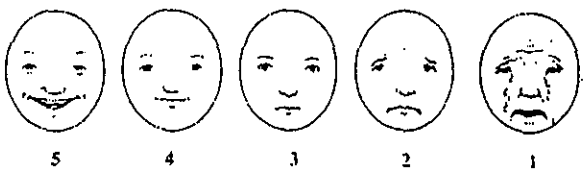

Figure 1. QOL Questionnaire for cancer patients treated with anticancer drugs.

In other words, no specific analytical techniques were applied to deal with missing data, such as imputation-based approaches (24,25). The frequencies of adverse events and deterioration of PS in week 4 (last week of the first course), however, were calculated to verify the assumption for the missing data.

These analyses were performed using SAS for Windows release 8.02 (SAS Institute, Cary, NC) and the general linear model analyses were carried out using the SAS Mixed procedure.

RESULTS

CLINICAL CHARACTERISTICS

Of 583 eligible patients randomized into the phase III trials, 377 who completed the questionnaire before treatment and at least once during the first course were analyzed. Their baseline clinical characteristics and treatment outcomes are summarized in Table 1. The median age was 61 years (range: 35–75

Table 1. Baseline characteristics and treatment outcomes of the study population

Patient characteristic	Study population (n = 377): No. (%)	Patients excluded from analysis* (n = 206): No. (%)
Age (years)		
<65	210 (55.7)	119 (57.8)
≥65 years	167 (44.3)	87 (42.2)
Median	61	61
Min., max.	35, 75	35, 75
Gender		
Male	285 (75.6)	161 (78.2)
Female	92 (24.4)	45 (21.8)
Stage		
IIIB	150 (39.8)	75 (36.4)
IV	227 (60.2)	131 (63.6)
PS		
0	97 (25.7)	43 (20.9)
1	265 (70.3)	145 (70.4)
2	15 (4.0)	18 (8.7)
Weight loss (%)		
≥5	74 (19.6)	58 (28.2)
<5	263 (69.8)	121 (58.7)
Unknown	40 (10.6)	27 (13.1)
Treatment exposure (No. of courses)		
1	53	52
2	159	87
3	118	33
4	41	23
5–6	6	2
Tumor response rate (%)	32.6	23.6
Median survival time (weeks)	52	37

*Patients who did not complete a questionnaire, including 18 and 38 patients who filled out the questionnaire only at baseline and only after the start of treatment, respectively.

years) and 78.5% of the patients were men. The patients had stage IV (61.4%) or stage IIIB (38.4%) disease, but a good PS (ECOG PS was 0–1 in 96.9%). These 377 patients had almost the same clinical characteristics at baseline as those patients who were excluded from the analysis. Higher tumor response rate, longer median survival time and better treatment compliance, however, were observed in the 377 analyzed patients compared with the excluded patients.

QOL and Clinical Parameters

The mean scores for the four domains calculated using the QOL data observed at baseline and at each week during the first course of chemotherapy are shown in Fig. 2. Marked changes in QOL scores were observed over time, especially for the physical and functional domains. The maximum decrease in score for each domain was observed at week 1 (within a week before day 8 of the first course). The compliance of patients with QOL assessment is also shown in Fig. 2. Overall, compliance decreased from 96.0% in week 1 to 84.6% in week 4. During the second course of chemotherapy, the same profiles were found for all four domains as in the first course. In other words, highly similar mean QOL scores were observed for each of the four domains in the corresponding weeks of the two courses (i.e. weeks 1 and 5, 2 and 6, 3 and 7, 4 and 8). For example, the mean QOL scores for the physical domain in each of weeks 1–8 were 49.9, 62.7, 66.9, 72.1, 52.6, 62.3, 65.9 and 71.2, respectively. However, this finding must be viewed with caution because the compliance during the second course declined considerably from 81.4% in week 5 to 64.5% in week 8.

The clinical parameters of the patients during the first course of chemotherapy are summarized in Table 2. More severe nausea/vomiting and anorexia were experienced in week 1, while diarrhea and fever were seen more frequently in week 2. A small number of patients experienced peripheral neuropathy during the first course. More severe deterioration of PS was observed in weeks 1 and 2, while a noticeable number of patients experienced improved PS in week 4. The longitudinal changes in QOL scores could be divided into two phases of deterioration and recovery that appeared to correspond to the occurrence of adverse events and changes in PS. There were no large differences in the clinical parameter profiles between patients who were analyzed and those who were excluded from analysis. For example, of the 58 patients excluded from analysis in week 4, 1.7, 13.8, 1.7, 20.7, 5.2 and 10.4% experienced nausea/vomiting, anorexia, diarrhea, fever, peripheral neuropathy and deterioration of PS, respectively. Grade 2 and 3 (and 4) toxicities observed were nausea/vomiting (grade 2, 0%; grade 3; 1.7%), anorexia (grade 2, 5.2%; grade 3, 1.7%); diarrhea (grade 2, 0%; grades 3 and 4, 1.7%) and fever (grade 2, 3.5%; grade 3, 0%). Grade 2 and 3 peripheral neuropathy did not occur in these 58 patients. Changes in more than one PS score were observed in 5.2% of patients.

Multivariate Longitudinal Data Analysis

The general linear model was used to evaluate the influence of clinical parameters on all four domains simultaneously. In the analysis, a negative regression coefficient indicated the occurrence of more severe adverse events related to the deterioration of QOL. Table 3 shows coefficients with P values that were estimated from the general linear model. Nausea/vomiting and anorexia had a significant influence on all four domains, especially the physical one. Diarrhea had a significant influence not only on the physical but also on the mental and psychosocial domains. However, influence of fever and peripheral neuropathy on the domains was not detected. PS was significantly related to the mental as well as functional and physical domains.

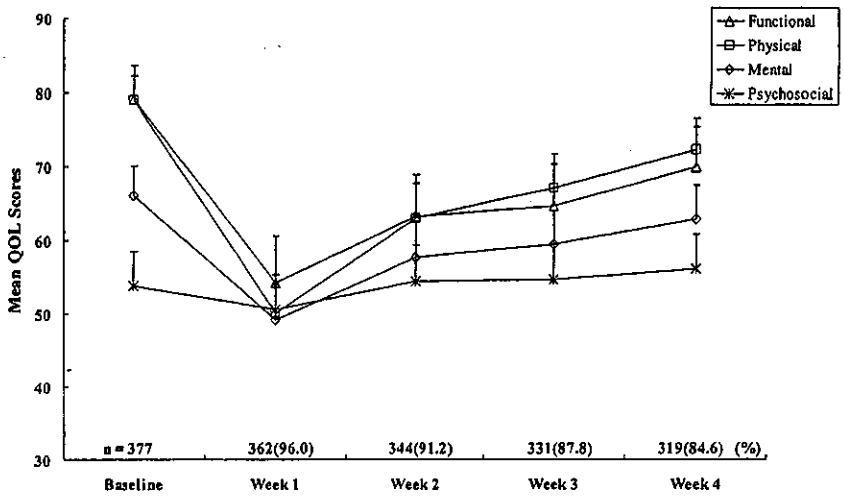

Figure 2. Mean QOL scores (+ 95% confidence intervals) for the functional, physical, mental and psychosocial domains at baseline (Baseline) and for each week of the first course (weeks 1–4).

Based on the results of subgroup analyses, neither qualitative nor noticeable quantitative differences were observed. In other words, similar relationships between clinical parameters and QOL domains were also observed in the gender, age and treatment arm subgroups.

DISCUSSION

In earlier studies, cross-sectional or one-dimensional QOL data were analyzed to investigate influences of chemotherapy-induced adverse events on QOL in different types of cancer (12–17). However, in order to capture the entire effect of chemotherapy-induced adverse events on QOL, it is important to assess QOL by using a multi-dimensional instrument (12). Moreover, repeated QOL assessments over time during chemotherapy may be vital, especially when anti-cancer drugs are administered on a weekly schedule. Thus, QOL data should be both longitudinal and multi-dimensional. In this study, we analyzed weekly-collected multi-dimensional QOL data during chemotherapy to determine the relative influence

Table 2. Profile of clinical parameters for every week (weeks 1 to 4) during the first course (% of patients)

Time	*n*	Nausea/vomiting		Anorexia		Diarrhea		Fever		Peripheral neuropathy		Change of PS*			
		≤G1	G2/G3	≤G1	G2/G3	≤G1	G2/G3	≤G1	G2/G3	≤G1	G2/G3	≤−1	0	1	≥2
Week 1	362	40.3	9.4/4.7	56.1	21.0/5.5	11.9	3.6/0.8	11.9	1.9/0.0	0.8	0.0/0.0	4.4	72.7	18.0	4.9
Week 2	344	20.1	3.5/1.5	32.6	9.6/2.6	14.8	6.4/2.0	17.4	3.5/0.3	1.7	0.0/0.0	6.4	73.3	16.0	4.3
Week 3	331	11.8	1.8/0.3	26.9	5.1/1.5	9.1	1.8/1.2	16.3	2.7/0.0	3.3	0.0/0.0	8.2	75.8	12.4	3.6
Week 4	319	3.8	0.9/0.0	12.9	2.8/0.0	2.2	0.3/0.3	6.9	0.6/0.0	3.5	0.6/0.0	12.9	76.2	9.1	1.9

Abbreviations: PS, performance status; G, grade. *Changes from baseline of PS: a positive value represents deterioration of PS.

Table 3. Relationship between clinical parameters and the four domains

Clinical parameter	Functional		Physical		Mental		Psychosocial	
	Reg. coeff.*	*P*-Value	Reg. coeff.*	*P*-Value	Reg. coeff.*	*P*-Value	Reg. coeff.*	*P*-Value
Nausea/vomiting	−2.4	0.039	−6.6	<0.001	−2.6	0.009	−2.2	0.002
Anorexia	−9.0	<0.001	−9.7	<0.001	−5.7	<0.001	−2.1	0.001
Diarrhea	−1.9	0.059	−4.4	<0.001	−2.1	0.011	−1.2	0.044
Fever	−1.6	0.131	−0.6	0.519	−1.2	0.176	−0.9	0.187
Peripheral neuropathy	−4.8	0.143	−4.5	0.150	−3.1	0.265	−1.6	0.439
Performance status	−4.8	<0.001	−3.7	0.001	−3.5	0.001	−1.2	0.123

*Regression coefficient: a negative value means the occurrences of more severe adverse events or PS deterioration related to the deterioration of QOL.

of physician-assessed clinical parameters on QOL in patients with advanced NSCLC. A general linear model, in which the inter-domain and time-series correlations were handled, was employed for the analysis.

Our study demonstrated that nausea/vomiting, anorexia and diarrhea had a greater influence on the physical domain of the questionnaire than other domains. Interestingly, these clinical parameters had a considerable influence over the mental and psychosocial as well as physical and functional domains. Moreover, the relationship between the severity of adverse events and deterioration in the four domains appeared to hold when subgroups were analyzed by gender, age and treatment arm. Therefore, these data suggest that supportive treatment for non-hematological adverse events, especially gastrointestinal, could provide improvement/maintenance of not only physical and functional well-being, but also overall QOL, including mental and psychosocial well-being during chemotherapy in this study population.

Our analysis did not detect a significant influence of fever and peripheral neuropathy on any of the four domains. This may be due to lower frequencies of these adverse events compared with others. Because peripheral neuropathy, in particular, is a chronic toxicity of chemotherapy, its influence on QOL may only be captured if relevant data are collected with good compliance in the second course and thereafter in future studies.

It is necessary to discuss the limitations of this study with regard to missing QOL data commonly observed in the context of advanced stage disease (26,27). First, 206 patients who did not complete the QOL questionnaires were excluded from the analysis. Whereas the baseline clinical characteristics of patients analyzed were very similar to those of the excluded patients, treatment outcomes, such as tumor response, survival time and treatment compliance were rather different, in favor of the subset of analyzed patients. Although we used the data gathered prior to the second course of chemotherapy to avoid possible impacts of disease progression or early termination of treatment on the associations between clinical parameters and QOL, we admit that the results from our analysis may not easily be generalized. Second, a noticeable number of patients (4, 9, 12 and 15% in weeks 1, 2, 3 and 4, respectively) did not fill out the QOL questionnaire, even during the first course of chemotherapy. Similar frequencies of adverse events and a similar deterioration in PS, however, were observed between the patients who filled out the questionnaires and those who did not. This result could support the validity of our findings, because the missing QOL data observed during the first course was probably unrelated to the clinical deterioration and therefore its impacts on our analysis may be ignored. Finally, we did not analyze QOL data obtained in the second course of treatment and thereafter because of the high percentage of missing assessments (for example, 35% at the end of the second course). Taking into account that the current standard chemotherapy has a tendency to be repeated for more than two courses, the influence of chemotherapy on QOL in the second course or later phases of treatment should be examined in future studies where the best efforts (better staff training and more support of clinical research coordinators) are made to improve data collection.

In conclusion, we analyzed multi-dimensional repeated QOL data and revealed significant influences of nausea/vomiting, anorexia and diarrhea on mental and psychosocial, as well as physical and functional, domains of patients with advanced NSCLC undergoing chemotherapy. These findings suggest that supportive care to control these non-hematological adverse events could help maintain overall QOL during chemotherapy in cancer patients.

Acknowledgments

We thank the West and East CPT-11 Lung Cancer Study Groups (Dr Hisanobu Niitani, Dr Masahiro Fukuoka, Dr Keiichi Nagao and other investigators) for providing us with valuable data. We also thank Akira Sahashi, Fumiyasu Fukuda and Tomoji Miyazaki for skillful data management during this study. This paper could not have been written without their helpful contributions.

References

1. Aaronson NK, Bullinger M, Ahmedzai S. A modular approach to quality-of-life assessment in cancer clinical trials. *Recent Results Cancer Res* 1988;111:231–49.
2. Aaronson NK, Ahmedzai S, Bergman B, Bullinger M, Cull A, Duez NJ, et al. The European Organization for Research and Treatment of Cancer QLQ-C30: a quality-of-life instrument for use in international clinical trials in oncology. *J Natl Cancer Inst* 1993;85:365–76.
3. Osoba D. Lessons learned from measuring health-related quality of life in oncology. *J Clin Oncol* 1994;12:608–16.
4. Moinpour CM, Feigl P, Metch B, Hayden KA, Meyskens FL, Crowley J. Quality of life end points in cancer clinical trials: reviews and recommendations. *J Natl Cancer Inst* 1989;81:485–95.
5. Gotay CC, Korn EL, McCabe MS, Moore TD, Cheson BD. Quality-of-life assessment in cancer treatment protocols: research issues in protocol development. *J Natl Cancer Inst* 1992;84:575–9.
6. Nayfield SG, Ganz PA, Moinpour CM, Cella DF, Hailey BJ. Report from a National Cancer Institute (USA) Workshop on quality of life assessment in cancer clinical trials. *Qual Life Res* 1992;1:203–10.
7. Machin D. Assessment of quality of life in clinical trials of the British Medical Research Council. *J Natl Cancer Inst Monogr* 1996;20:97–102.
8. Ganz PA. Quality of life and the patient with cancer: individual and policy implications. *Cancer* 1994;74(Suppl 4):1445–52.
9. Rowland JH. Outcome assessment: cancer-specific quality-of-life measures – beyond the research setting. In: Educational Book, ASCO 33rd Annual Meeting, Denver, CO, May 17–20, 1997;342–9.
10. Cella DF, Tulsky DS, Gray G, Sarafian B, Linn E, Bonomi A, et al. The functional assessment of cancer therapy scale: development and validation of the general measure. *J Clin Oncol* 1993;11:570–9.
11. Kurihara M, Shimizu H, Tsuboi K, Kobayashi K, Murakami M, Eguchi K, et al. Development of quality of life questionnaire in Japan: quality of life assessment of cancer patients receiving chemotherapy. *Psycho-Oncology* 1999;8:355–63.
12. Osoba D, Zee B, Warr D, Kaizer L, Latreille J, Pater J. Quality of life studies in chemotherapy-induced emesis. *Oncology* 1996;53(Suppl 1): 92–5.
13. Lindley CM, Hirsch JD, O'Neill CV, Transau MC, Gilbert CS, Osterhaus JT. Quality of life consequences of chemotherapy-induced emesis. *Qual Life Res* 1992;1:331–40.
14. O'Brien BJ, Rusthoven J, Rocchi A, Latreille J, Fine S, Vandenberg T, et al. Impact of chemotherapy-associated nausea and vomiting on patients' functional status and on costs: survey of five Canadian centres. *Can Med Assoc J* 1993;143:296–302.

15. Luoma ML, Hakamies-Blomqvist L, Sjostrom J, Mouridsen H, Pluzanska A, Malmstrom P, et al. Physical performance, toxicity and quality of life as assessed by the physician and the patient. *Acta Oncol* 2002;41:44–9.
16. Harper-Wynne C, English J, Meyer L, Bower M, Archer C, Sinnett HD, et al. Randomized trial to compare the efficacy and toxicity of cyclophosphamide, methotrexate and 5-fluorouracil (CMF) with methotrexate mitoxantrone (MM) in advanced carcinoma of the breast. *Br J Cancer* 1999;81:316–22.
17. Tchekmedyian NS, Hickman M, Siau J, Greco A, Aisner J. Treatment of cancer anorexia with megestrol acetate: impact on quality of life. *Oncology* 1990;4:185–92.
18. Diggle PJ, Heagerty P, Liang KY, Zeger SL. Analysis of Longitudinal Data, 2nd ed. Oxford: Oxford, Oxford University Press 2002:22–80.
19. Matsumoto T, Ohashi Y, Morita S, Kobayashi K, Shibuya M, Yamaji Y, et al. QOL questionnaire for cancer patients treated with anticancer drugs (QOL-ACD): validity and reliability in Japanese patients with advanced non-small cell lung cancer. *Qual Life Res* 2002;11:483–93.
20. World Health Organization. WHO Handbook for Reporting Results of Cancer Treatment. WHO Offset Publication No. 48. Geneva: WHO 1979.
21. Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982;5:649–55.
22. Galecki AT. General class of covariance structures for two or more repeated factors in longitudinal data analysis. *Commun Statist* 1994;23:3105–19.
23. Littell R, Milliken G, Stroup W, Wolfinger RD. SAS System for Mixed Models. Cary, SC: SAS Institute 1996;87–134.
24. Staquet MJ, Hays RD, Fayers PM. Quality of Life Assessment in Clinical Trials. Oxford: Oxford University Press 1998;227–80.
25. Curran D, Molenberghs G, Fayers PM, Machin D. Incomplete quality of life data in randomized trials: missing forms. *Stat Med* 1998;17:697–709.
26. Bernhard BJ, Cella DF, Coates AS, Fallowfield L, Ganz PA, Moinpour CM, et al. Missing quality of life data in cancer clinical trials: serious problems and challenges. *Stat Med* 1998;17:517–32.
27. Moinpour CM, Lyons B, Grevstad PK, Lovato LC, Crowley J, Czaplicki K, et al. Quality of life in advanced non-small cell lung cancer: results of a Southwest Oncology Group randomized trial. *Qual Life Res* 2002;11:115–26.

International Immunology Advance Access published May 4, 2004

International Immunology 2004; Page 1 of 7
doi: 10.1093/intimm/dxh082

Unexpected role of TNF-α in graft versus host reaction (GVHR): donor-derived TNF-α suppresses GVHR via inhibition of IFN-γ-dependent donor type-1 immunity

Satoshi Yamamoto[1,2], Takemasa Tsuji[1], Junko Matsuzaki[1], Yue Zhange[1], Kenji Chamoto[1], Akemi Kosaka[1], Yuji Togashi[1], Kenji Sekikawa[3], Ken-ichi Sawada[2], Tsuguhide Takeshima[1], Takao Koike[2] and **Takashi Nishimura[1]**

[1]Division of Immunoregulation, Institute for Genetic Medicine and [2]Department of Internal Medicine II, School of Medicine, Hokkaido University, Sapporo, Japan
[3]National Institute of Animal Health, Tsukuba, Japan

Keywords: CTL, cytokines, graft vs host disease, Th1/Th2 cells, transplantation

Abstract

Graft versus host disease (GVHD) is a major complication of allogeneic hematopoietic stem cell transplantation, leading to significant morbidity and mortality. Host-derived TNF-α play a role in the induction of allo-reactive donor T cell activation and the pathogenesis of GVHD. On the other hand, the precise role of donor-derived TNF-α in GVHD remains unclear. To elucidate this issue, we designed an acute GVHD model using (B6×D2) F1 recipient mice transferred with spleen cells derived from either wild-type or TNF-α$^{-/-}$ C57BL/6 mice. Surprisingly, we found that spleen cells from TNF-α$^{-/-}$ mice induce more severe graft versus host reaction (GVHR) than wild-type spleen cells upon transfer into B6D2F1 mice. Transplantation of TNF-α$^{-/-}$ mouse spleen cells was associated with enhanced anti-host CTL generation and augmented deletion of host cells. Moreover, mice receiving TNF-α$^{-/-}$ cells showed significantly higher levels of serum IFN-γ, which was mainly produced by donor CD8^{+} T cells. We also demonstrated that TNF-α deficiency in donor spleen cells caused a marked elevation of TNF-α producing capacity by LPS-stimulated host macrophages. Such enhanced GVHR was completely prevented by using TNF-α$^{-/-}$IFN-γ$^{-/-}$ splenic cells. Our findings demonstrate, for the first time, that donor-derived TNF-α suppress GVHR by inhibiting IFN-γ-dependent donor type-1 immunity which is essential for host TNF-α elevation.

Introduction

Allogeneic hematopoietic stem cell transplantation (HST) has been an effective treatment of hematologic malignancies and genetic disorders (1). The success rate of HST has steadily increased in recent years, but graft versus host disease (GVHD) is still a major cause of post-transplantation mortality (2,3). The generation of a strong graft versus host reaction (GVHR) is induced by activated donor T cells which recognize major and/or minor histocompatibility Ag mismatches. Cytokine dysregulation and organ damage due to pre-transplantation conditioning regimens are also involved in the development of GVHR (4–7).

Tumor necrosis factor α (TNF-α) has been implicated in the pathogenesis of GVHD. TNF-α induces a direct toxicity to host tissues and enhances the expression of MHC (8) and adhesion molecules (9). Moreover TNF-α may act as an autocrine T cell growth factor (10) and thus augment donor T cell clonal expansion. Anti-TNF-α mAb can ameliorate the severity of GVHD (11–13). In a recent study, it was shown that TNF-αR p55 of the recipient controls early GVHD (14) and that TNF-αR p55 of the donor plays a critical role in allo-reactive T cell response (15). Therefore, recipient-derived TNF-α contributes to the activation of allo-reactive T cells and augments the severity of acute GVHD. In contrast, the role of donor-derived TNF-α in GVHD remains unclear.

In the present study, we have examined the role of donor-derived TNF-α in allo-reactive T cell responses *in vivo* using well-characterized mouse models of GVHD. We demonstrate that mice transferred with TNF-α-deficient mouse spleen cells

Correspondence to: T. Nishimura; E-mail: tak24@igm.hokudai.ac.jp

Transmitting editor: T. Saito

Received 9 January 2004, *accepted* 4 March 2004

exhibit augmented general parameters associated with GVHR, including early elevation of donor-derived IFN-γ, generation of anti-host CTL and producing host-derived TNF-α. These data document an unrecognized role of donor-derived TNF-α, which might suppress early GVHR through the control of IFN-γ-dependent donor type-1 immunity.

Methods

Mice

C57BL/6J (B6) (H-2^b) and B6 × DBA/2 F1 (B6D2F1) mice were obtained from Charles River Japan (Yokohama, Japan), TNF-$\alpha^{-/-}$ C57BL/6 mice were provided by Dr K. Sekikawa (Department of Immunology, National Institute of Animal Health, Tsukuba, Japan) and IFN-$\gamma^{-/-}$ C57BL/6 mice were provided by Dr Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). 6–10 week-old mice were used for all experiments.

Induction of GVHD

Single-cell suspensions from the spleens of B6 and B6D2F1 mice were prepared in RPMI 1640 medium (Gibco-BRL, Grand Island, NY). The cells were suspended in PBS. Acute GVHD was induced by injecting B6 spleen cells (5×10^7) into B6D2F1 mice. Age-matched B6D2F1 mice transferred with syngeneic spleen cells (5×10^7) were used as control mice.

Flow cytometric analysis

The phenotypic characterization of spleen cells by flow cytometry was carried out using a FACSCalibur instrument and CELLQuest software (Becton Dickinson, San Jose, CA). mAbs used in these experiments [phycoerythrin (PE)-conjugated anti-CD4 mAb, PE-conjugated anti-CD8 mAb, PE-conjugated anti-B220 mAb and fluorescein isothocyanate (FITC)-conjugated H-2K^d mAb] were purchased from PharMingen (San Diego, CA).

Intracellular cytokine expression

For the detection of cytoplasmic cytokine expression, cells stimulated with immobilized anti-CD3 mAb for 6 h in the presence of Brefeldin A were first stained with PerCP-conjugated anti-CD4 mAb or cychrome-conjugated anti-CD8 mAb and FITC-conjugated anti-H-2K^d mAb, fixed with 4% paraformaldehyde and treated with permeabilizing solution (50 mM NaCl, 5 mM EDTA, 0.02% NaN_3 and 0.5% Triton X-100, pH 7.5). The fixed cells were then stained with PE-conjugated anti-IFN-γ mAb for 45 min on ice. The percentage of cells expressing cytoplasmic IFN-γ was determined by flow cytometry (FACSCalibur). PerCP-conjugated anti-CD4 mAb, cytochrome-conjugated anti-CD8 mAb and PE-conjugated anti-IFN-γ mAb were purchased from PharMingen.

Cytotoxicity assay

The cytotoxicity mediated by CTL was measured by 4 h ^{51}Cr-release assay as described previously (16). H-2K^d specific cytotoxicity was determined using DBA/2-derived P815 mastcytoma cells (H-2K^d) as target cells. As control, C57BL/6-derived MBL-2 T lymphocyte cells (H-2K^b) were used. The percentage cytotoxicity was calculated as described previously (16).

Generation of CTL in mixed lymphocyte culture (MLC)

Spleen cells (5×10^6 cells) from GVHD mice and control mice were co-cultured with BDF1 mouse spleen cells (2.5×10^6 cells) which were inactivated by pretreatment with mitomycin C (60 μg/ml; Kyowa Hakko Kogyo, Tokyo, Japan). Cells were co-cultured for 4 days in flat-bottomed 12-well plates. After culture, cells were harvested and their cytotoxicity was measured.

Measurement of cytokine levels by ELISA

IFN-γ levels in serum or culture supernatants were evaluated with commercial ELISA kit (Amersham International, Buckinghamshire, UK) according to the manufacturer's instructions.

Measurement of serum TNF-α levels induced by LPS injection

LPS-induced TNF-α production was assayed in mice transferred with spleen cells from wild-type or TNF-$\alpha^{-/-}$ mouse spleen cells. As a control, B6D2F1 mice transferred with syngeneic mouse spleen cells were used. Ten days after GVHR induction, the mice were treated with or without i.v. injection of LPS (10 μg) and their serum samples were harvested 90 min after LPS injection to determine serum TNF-α levels by ELISA (Amersham).

Statistical analysis

Difference between the means of experimental groups were analyzed using the Student's *t*-test. Differences were considered significant where $P < 0.05$.

Results

TNF-α deficiency in donor cells accelerates GVHR in mice

B6D2F1 (H-$2^{b,d}$) mice were treated with i.v. injection of wild-type or TNF-$\alpha^{-/-}$ C57BL/6 (H-2^b) spleen cells. As a control, mice were injected with syngeneic B6D2F1 spleen cells. After 14 days, mice were sacrificed to examine the frequency of host cell deletion, as detected with anti-H-2^d mAbs and flow cytometry. As shown in Fig. 1, in mice transferred with wild-type C57BL/6 mouse spleen cells the percentage of host B cells decreased to 28.7%. Deletion of host B cells was further enhanced (84.3%) when TNF-$\alpha^{-/-}$ mouse spleen cells were transferred into B6D2F1 mice. Host cell deletion was also demonstrated among CD4$^+$ and CD8$^+$ T cells (data not shown). Consistent with these findings, spleen cells obtained from B6D2F1 mice treated with TNF-$\alpha^{-/-}$ splenocytes exhibited higher levels of anti-host CTL activity compared with spleen cells from mice transferred with control (B6D2F1) or wild-type C57BL/6 mouse splenocytes (Fig. 2).

TNF-α deficiency in donor cells accelerates the elevation of serum IFN-γ levels initiated by donor type-1 immunity during GVHD

As previously described (17,18), type-1 cytokines such as IL-12 and IFN-γ play a critical role in acute GVHD induction.

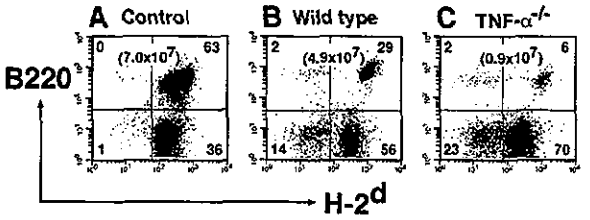

Fig. 1. Acceleration of host B cell deletion by GVH response in mice transferred with TNF-$\alpha^{-/-}$ spleen cells. GVH response was induced in BDF1 mice by cell transfer with (A) syngeneic BDF1 (H-$2^{b,d}$) spleen cells, (B) wild-type C57BL/6 (H-2^{b}) spleen cells or (C) TNF-$\alpha^{-/-}$ C57BL/6 (H-2^{b}) spleen cells as described in Methods (n = 6). Fourteen days after GVH induction, host B cell deletion by GVHD response was determined by flow cytometry after staining with PE-labeled anti-B220 mAb and FITC-labeled anti-H-2K^{d} mAb. The numbers represent the percentage of cells in spleen cells. The total cell numbers of B cells in spleen is indicated in parentheses. Similar results were obtained in three different experiments.

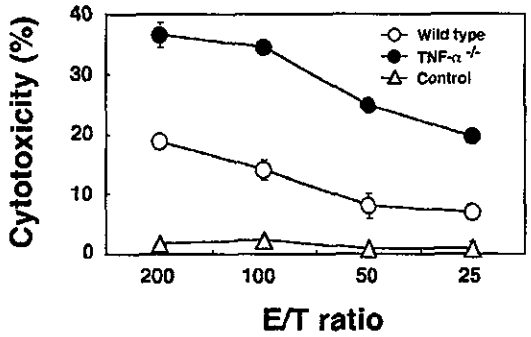

Fig. 2. Augmentation of anti host CTL generation in mice transferred with TNF-$\alpha^{-/-}$ spleen cells. BDF1 recipient mice were transferred with BDF1 mouse spleen cells (closed triangle), wild-type C57BL/6 (open circle) or TNF-$\alpha^{-/-}$ C57BL/6 (closed circle) spleen cells. After 14 days, spleen cells were harvested from all mice and their CTL activity against host type P815 mastocytoma cells was measured by 4 h ^{51}Cr-release assay. The data represent mean ± SE of three mice. Similar results were obtained in three different experiments.

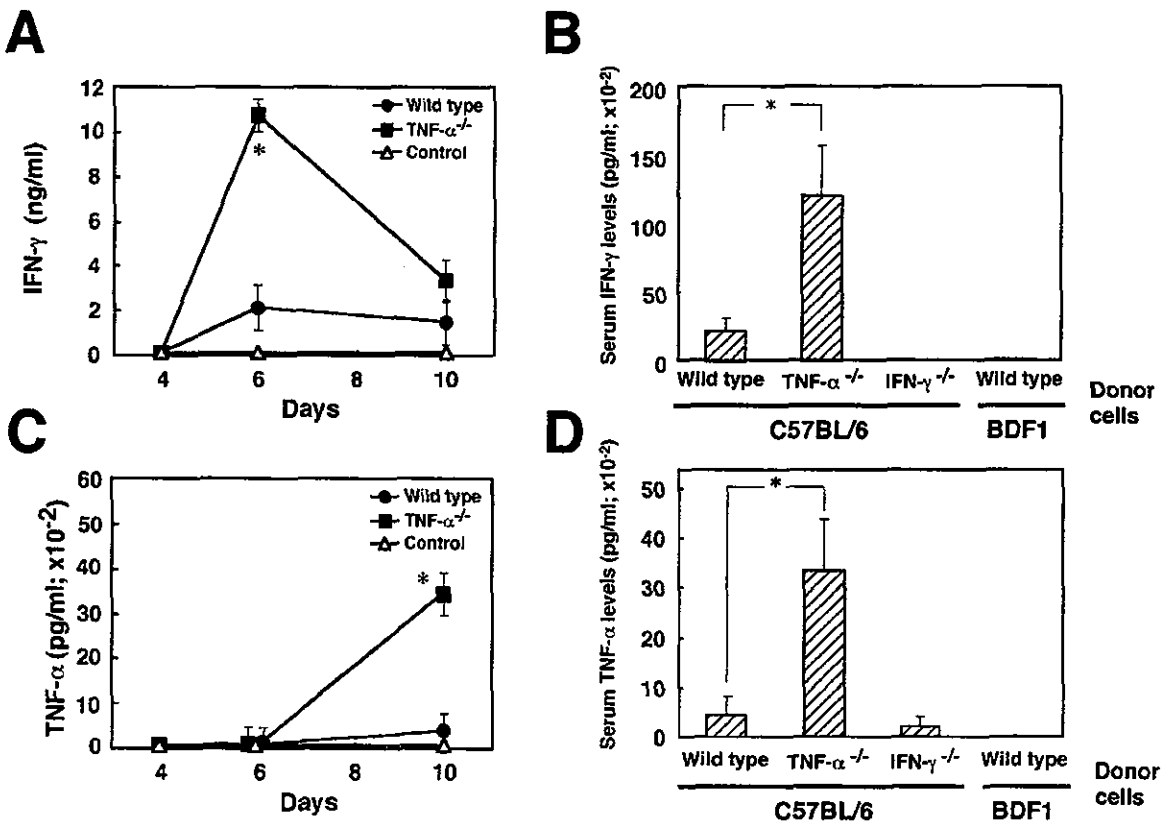

Fig. 3. TNF-$\alpha^{-/-}$ mice exhibited augmented producing ability of IFN-γ and TNF-α during GVHD. (A and B) Serum IFN-γ levels induced by allogeneic TNF-$\alpha^{-/-}$ spleen cells during GVHD. BDF1 recipient mice were transferred with syngeneic BDF1, wild-type C57BL/6, TNF-$\alpha^{-/-}$ C57BL/6 or IFN-$\gamma^{-/-}$ C57BL/6 spleen cells. (A), After 2, 6 or 10 days, serum IFN-γ levels of all mice were measured by ELISA. (B) Serum IFN-γ levels 6 days after donor cell transfer. (C and D) LPS-induced TNF-α production in recipient mice transferred with allogeneic TNF-$\alpha^{-/-}$ spleen cells. BDF1 recipient mice were transferred with syngeneic BDF1, wild-type C57BL/6, TNF-$\alpha^{-/-}$ C57BL/6 or IFN-$\gamma^{-/-}$ spleen cells. (C) After 2, 6 or 10 days, the recipient mice were treated with i.v. injection of LPS (10 μg/mouse) and their serum TNF-α level was determined by ELISA 90 min after LPS injection. (D) LPS-induced serum TNF-α elevation 10 days after donor cell transfer. The data represent mean ± SE of three mice. Similar results were obtained in three different experiments. *$P < 0.05$.