Table 1. Laboratory data on admission

ESR	39 mm/h	Blood chemistry		Immunological	
Urinalysis		TP	7.0 g/dl	IgG	1880 mg/dl
Protein	(-)	Alb	3.6 g/dl	IgA	501 mg/dl
Sugar	(-)	BUN	12.3 mg/dl	IgM	245 mg/dl
Cast	(-)	Cre	0.6 mg/dl	CRP	0.24 mg/dl
CBC		Ca	9.2 mg/dl	C3	65 mg/dl
WBC	4400/μl	IP	3.1 mg/dl	C4	17 mg/dl
	(Band+Seg 65, Lymph 20, Mono 9, Eosino 5, Baso 1)	LDH	218 IU/l	IC (Anti-C3d)	11.8 μg/ml
RBC	$4.02 \times 10^6/\mu l$	ALT	36 IU/l	RF	<10 IU/ml
Hb	12.2 g/dl	AST	31 IU/l	ANA	(-)
Ht	37.2 %	CK	48 IU/l	Cryoglobulin	(-)
Plt	$25.2 \times 10^4/\mu l$	FBS	91 mg/dl	PR3-ANCA	(-)
		ACE	23.6 IU/l	MPO-ANCA	(-)
		Lysozyme	12.5 μg/ml	Anti-dsDNA	(-)
		Vitamin B_{12}	1090 pg/dl (233–914)	Anti-U1RNP	(-)
				Anti-SSA	(-)
				Anti-SSB	(-)
				Anti-Jo-1	(-)

IC, immune complex

Table 2. Nerve conduction studies

	June 2000	January 2001	January 2003
Median nerve (rt)			
DLT (ms)	6.8	4.2	4.7
CMAP (mV)	4.0	9.9	13.4
MCV (m/s)	50.0	48.7	47.3
SNAP(μV)	12	18	49
SCV (m/s)	29.2	42.4	42.4
Ulnar nerve (rt)			
DLT (ms)	5.2	3.7	4.4
CMAP (mV)	7.0	17	18
MCV (m/s)	52.5	52.5	48.6
Tibial nerve (rt)			
DLT (ms)	N.E.	5.1	4.1
CMAP (mV)	N.E.	4.0	15.2
MCV (m/s)	N.E.	38.6	43.7
Peroneal nerve (rt)			
DLT (ms)	5.3	4.8	5.4
CMAP (mV)	0.2	–	64
MCV (m/s)	42.8	40.7	41.7

DLT, distal latency time; CMAP, compound muscle action potential; MCV, motor conduction velocity; SNAP, sensory nerve action potential; SCV, sensory conduction velocity; N.E., not evoked

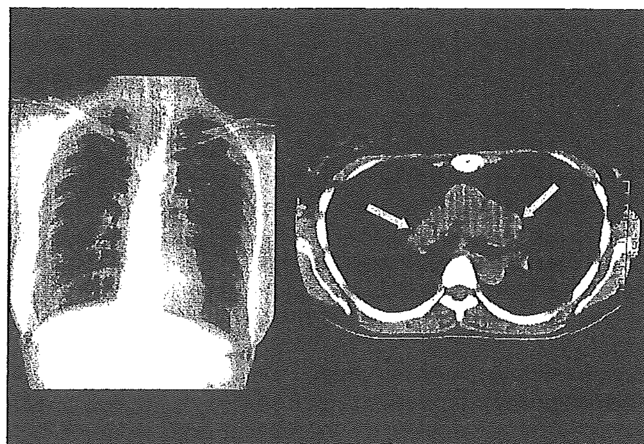

Fig. 1. Chest computed tomography findings on admission. Bilateral hilar lymphadenopathy was present (*arrows*)

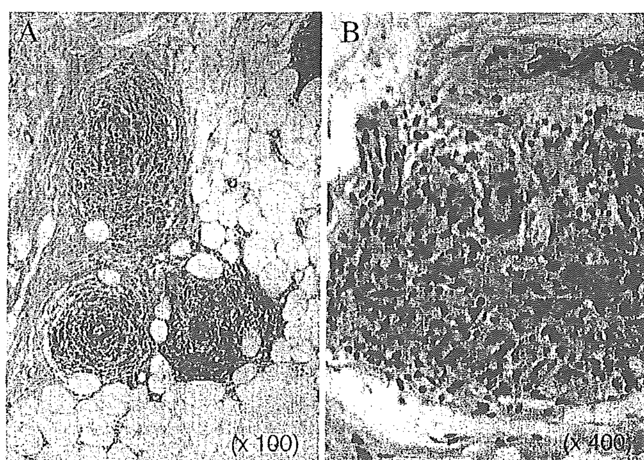

Fig. 2A,B. Histological sections of the erythematous dermal biopsy specimen (H&E staining; **A** ×100, **B** ×400). In higher magnification, non-caseating granuloma with infiltrating lymphocytes and a few giant cells are noted

in all nerves tested. In needle electromyography, denervation potentials were observed in the distal muscles. These findings were compatible with sensorimotor polyneuropathy due to axonal degeneration rather than segmental demyelination.

Slit-lamp biomicroscopy revealed that the patient had uveitis. Swelling of bilateral hilar lymph nodes was observed by chest computed tomography (Fig. 1), and gallium scintigraphy disclosed abnormal uptake of bilateral hilar lymph nodes that was consistent with the finding of active sarcoidosis. The dermal biopsy of leg erythema showed non-caseating granuloma with infiltration of lymphocytes and a few giant cells, but no eosinophils (Fig. 2). Diseases possibly causing peripheral neuropathy due to axonal

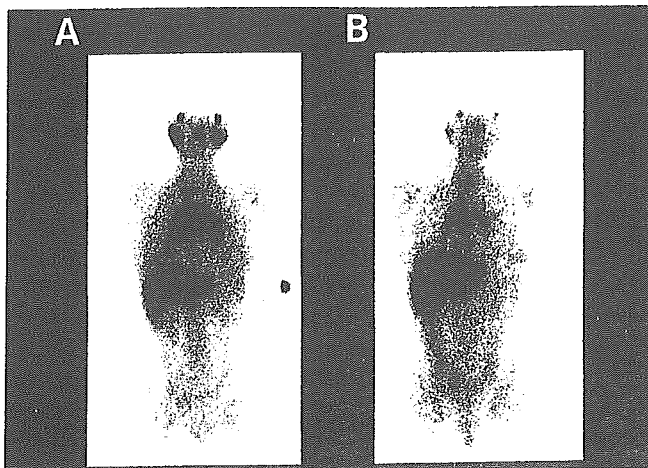

Fig. 3. Gallium scintigraphy of the whole body before (**A**) and after (**B**) steroid treatment. Abnormal uptake in the bilateral hilum improved markedly after treatment

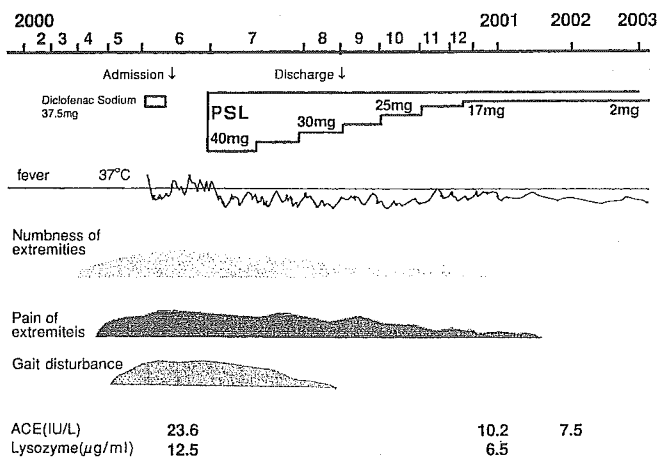

Fig. 4. Clinical course of this case. After prednisolone (*PSL*) treatment, numbness and pain of the extremities and gait disturbance were improved. Serum levels of angiotensin-converting enzyme (*ACE*) and lysozyme were also decreased

degeneration, such as metabolic diseases, toxic diseases, chronic inflammatory demyelinating polyneuropathy, infectious diseases, Vitamin B_{12} deficiency, and other collagen diseases, were all absent.

In July 2000, the diagnosis of sarcoidosis was made and the patient was started on 40mg daily of prednisolone (PSL), resulting in partial improvement of numbness and painful difficulty in walking. The following nerve conduction studies in January 2001 and January 2003 showed a marked improvement in CMAPs in the median, ulnar, tibial, and peroneal nerves, and SNAPs in the median nerve (Table 2). Abnormal uptake in the bilateral hilum also improved greatly after treatment (Fig. 3). Although the PSL dose was tapered gradually, her symptoms of peripheral neuropathies have been well controlled for 3 years. She continued to take the low dose of PSL (2mg daily) with no severe adverse events (Fig. 4).

Discussion

This is a case of sarcoidosis that showed sensorimotor polyneuropathy as an initial clinical manifestation. Neurological involvement in sarcoidosis has been reported to occur in 5%–15% of cases.[1,2] Moreover, in the context of neurological involvement, the prevalence of peripheral neuropathy including cranial nerve abnormality is estimated to occur in 4%–14% of cases,[3] although a recent study reported a higher incidence of peripheral nerve involvement.[4]

Previous studies have reported similar cases of sarcoidosis indicating spinal peripheral neuropathy (Table 3).[5-13] Of these, clinical features of 16 cases, including ours, are available for comparison. Ten of these 16 patients (63%) presented with peripheral neuropathy associated with sarcoidosis as an initial manifestation.[5-10,13] Thirteen (81%) cases had pulmonary symptoms during their course.[5,7-9,11-13] All patients were given corticosteroid therapy with improvement of their symptoms with only one exception. The patterns of neuropathy were variable, as seen in the previous studies.[4,6] Eight of 16 (50%) had sensorimotor polyneuropathy, four (25%) multifocal sensorimotor neuropathy, three (19%) multifocal sensory neuropathy, and one (6%) multifocal motor neuropathy.

The previous reports[1,6,7,11,12] indicated that sarcoidosis could elicit both compressive neuropathy due to perineural granuloma formation and ischemic neuropathy due to periangitis. Typically, complete compression causes Wallerian degeneration followed by demyelination, while vasculitis induces segmental demyelination first because Schwann cells are more vulnerable to ischemia. Although we did not perform sural nerve biopsy, the electrophysiological findings suggested that the main mechanism involved in this case was axonal degeneration. It is likely that sarcoid nodules observed in the specimen of skin biopsy compress the myelinated and unmyelinated neural fibers. Moreover, granulomatous vasculitis or vessel occlusion due to granulomas might also be involved in the neurological manifestations. In general, neuropathies due to vasculitis indicate mononeuritis multiplex. However, symmetrical polyneuropathy was also seen in previous reports.[5-7,14] Our case suggested symmetrical sensorimotor polyneuropathy. This might have been due to the severity and duration of the disease or the effects of systemic inflammation causing vasculitis.

We were able to perform nerve conduction tests before and after treatment. The electrophysiological parameters showed improvement after treatment. This suggests that nerve conduction studies are useful for evaluating the efficacy of treatment even when marked improvement of physiological symptoms is not seen.

Corticosteroid therapy is recommended for the peripheral neuropathy of sarcoidosis and is effective in most patients, although a placebo-controlled double-blind trial has not been performed. The improvement of electrophysiological parameters might be a consequence of the decreased ischemia due to vasculitis as well as reduction of

Table 3. Clinical manifestation of published neurosarcoidosis only manifesting the spinal peripheral neuropathy

First author/year[Ref.]	Age (years)/ sex	Initial manifestation	Pattern of neuropathy	Other symptoms	Therapy	Efficacy of PSL
Oh/1979[5]	58/F	Peripheral nerve	Sensorimotor polyneuropathy	Lung	100 mg	(+)
Nemni/1981[6]	29/F	Peripheral nerve	Sensorimotor polyneuropathy	(-)	150 mg every 2 days	(+)
Galassi/1984[7]	70/M	Peripheral nerve	Sensorimotor polyneuropathy	Lung	100 mg every 2 days	(+)
	54/M	Peripheral nerve	Sensorimotor polyneuropathy	Lung	High dose	(+)
Okada/1986[8]	25/M	Peripheral nerve Skin	Multifocal sensory neuropathy	Lung/skin/eye	60 mg every 2 days	(+)
Yamane/1986[9]	53/F	Peripheral nerve Skin	Sensorimotor polyneuropathy	Lung	40 mg	(-)
Krendel/1992[10]	39/F	Peripheral nerve Skin	Sensorimotor polyneuropathy	Skin	40 mg	(+)
Iwata/1993[11]	58/F	Lung	Multifocal sensory neuropathy	Lung/skin/eye	30 mg	(+)
Sharma/1996[12]	40/M	N.A.	Sensorimotor polyneuropathy	Lung/skin/heart	(+)	(+)
	33/M	N.A.	Multifocal motor neuropathy	Lung/skin/eye	(+)	(+)
	48/M	N.A.	Multifocal sensory neuropathy	Lung/lymph node	(+)	(+)
	50/M	N.A.	Multifocal sensory neuropathy	Lung	(+)	(+)
	46/F	N.A.	Multifocal sensory neuropathy	Lung/skin	(+)	(+)
Said/2002[13]	63/M	Peripheral nerve	Multifocal sensorimotor neuropathy	Lung	1 mg/kg	(+)
	69/M	Peripheral nerve	Multifocal sensorimotor neuropathy	(-)	(+)	(+)
Present study	45/F	Peripheral nerve Skin	Sensorimotor polyneuropathy	Lung/skin/eye	40 mg	(+)

N.A., not available

compression injury due to resolution of granulomatous lesions.

Sarcoidosis shows a wide variety of clinical features and its diagnosis is difficult in the absence of clinical manifestations such as cutaneous or pulmonary involvement. Any of the preceding neurologic manifestations can occur without any evidence of systemic features of sarcoidosis.[5-7,9,10] It is therefore important to consider the possibility of systemic disease including sarcoidosis even when only peripheral neuropathy is found.

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ORIGINAL ARTICLE

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Therapeutic effects of the combination of methotrexate and bucillamine in early rheumatoid arthritis: a multicenter, double-blind, randomized controlled study

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Abstract Disease-modifying antirheumatic drug (DMARD) combination therapies are used widely, but there have been few reports clearly demonstrating that combination therapy is more effective than DMARD monotherapy. We conducted a multicenter, double-blind controlled trial in order to clarify that the combination of methotrexate and bucillamine is more effective than either alone. The subjects of this study were 71 patients with active rheumatoid arthritis within 2 years of onset. Dosages were 8 mg methotrexate with 5 mg folic acid per week (MTX group), 200 mg bucillamine per day (BUC group), or both MTX and BUC (combination group). Clinical effects and adverse reactions were observed for 96 weeks. The ACR 20 response rate was 79.2% in the combination group, significantly higher than the rates of 43.5% for the MTX group ($P = 0.008$) and 45.8% for the BUC group ($P = 0.0178$). The cumulative survival curve of maintaining the ACR 20 response was significantly higher in the combina-

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tion group than in the MTX and BUC groups ($P = 0.0123$ and $P = 0.0088$, respectively). The mean increase in the total Sharp score over 96 weeks was 12.6 ± 9.0 in the combination group, significantly lower ($P = 0.0468$) than the value of 28.0 ± 28.3 for the single DMARD (combined MTX and BUC) group. The incidence of adverse reactions did not differ significantly between the three groups. It was concluded that the combination therapy with MTX and BUC showed significantly higher clinical efficacy than either of the single DMARD therapies.

Key words Bucillamine · Combination therapy · Methotrexate · Rheumatoid arthritis

Introduction

Although many studies have investigated the usefulness of combinations of disease-modifying antirheumatic drugs (DMARDs), very few studies have shown that a combination of two conventional DMARDs is more effective than the individual drugs alone.[1] For example, two double-blind controlled studies performed over 52 weeks showed no statistically significant increase in the disease amelioration rate with a combination of methotrexate (MTX) and sulfasalazine (SSZ).[2,3] Other combinations of DMARDs have also been investigated in double-blind controlled trials, but none of the combination therapies have been shown to be more effective than monotherapy.[4–7]

It was reported that the addition of cyclosporine was effective in subjects with inadequate control of rheumatoid activity by MTX.[8] As there was no comparison with treatment by cyclosporine alone, it was unclear, however, whether the effect of the combination therapy was the effect of the combination of MTX and cyclosporine, or the effect of cyclosporine itself. Several studies have also shown that the addition of a second conventional DMARD is effective in subjects with inadequate response to MTX,[9–11] but these are not thought to show exactly that combination therapies are more useful than monotherapy, as no comparisons were made of the effects of the combination and the additional DMARD alone. On the other hand, in comparison with MTX treatment alone, the combination of SSZ and hydroxychloroquine (HCQ), and the combination of these three drugs, O'Dell et al. found that simultaneous administration of the three DMARDs is the most effective.[12] Their study confirmed an additive or synergistic effect for this specific combination of DMARDs. There has, however, been no study showing by double-blind controlled trial that combination therapy with two conventional DMARDs is more effective than either DMARD administered as monotherapy.

In the present study, efficacy and adverse reactions were compared between MTX monotherapy, bucillamine (BUC) monotherapy, and the combination of both drugs, using a double-blind controlled trial, with the combination demonstrated to be the most effective. This is the first report showing that combination therapy with two conventional DMARDs is more effective than either DMARD administered as monotherapy.

Subjects and methods

The subjects were 71 Japanese patients aged from 20 to 70 years, within 2 years of the onset of continuous arthritic pain, who met the American College of Rheumatology (ACR) classification criteria for rheumatoid arthritis as revised in 1987. The subjects had 6 or more painful joints out of 48 and 3 or more swollen joints out of 46, and showed at least a 30 mm/h erythrocyte sedimentation rate (ESR), or 1.0 mg/dl or more of C-reactive protein (CRP). They had never received MTX or BUC treatment, or corticosteroids at dosages of 7.5 mg/day or more of prednisolone equivalent.

Twenty-three medical institutions participated in this study, and subjects entered the trial between February 1999 and March 2000. Subjects were divided into three groups, respectively administered MTX, BUC, or the combination of both drugs. After confirming a subject's eligibility for the study by telephone, one of the test drugs was assigned randomly according to the usage number for the test drug distributed beforehand to each institute. A placebo indistinguishable in appearance from the drug was prepared for MTX and BUC for a double dummy medication technique. Dosages were as follows: MTX 4 mg/week and BUC 100 mg/day for the first 4 weeks, then 6 mg/week and 200 mg/day respectively for the following 8 weeks, and 8 mg/week and 200 mg/day respectively from week 13 to week 96, the end of the study period. Folic acid 5 mg was administered 48 h after MTX or its placebo. The trial was discontinued for subjects who had not reached an ACR 20 response by 24 weeks, or who did not meet the ACR 20 response criteria thereafter. After withdrawal from the trial due to inadequate response, adverse reactions, etc., subsequent treatment was at the discretion of the treating physician. Planned observations, and laboratory and X-ray investigations were continued as scheduled until the end of the study period for these subjects.

The following observations and investigations were carried out at intervals of 4 weeks for each subject: physical examination, full blood count, hepatic transaminases, serum creatinine, blood urea nitrogen, and urinalysis. The following indices were determined to evaluate treatment effects just prior to study commencement and every 12 weeks thereafter for 98 weeks: number of painful joints, number of swollen joints, pain estimation by the subjects using the visual analogue scale (VAS), subject's global assessment of disease activity using the VAS, physician's overall assessment of disease activity using the VAS, the modified health-assessment questionnaire (mHAQ),[14] erythrocyte sedimentation rate (ESR) using the Westergren method, C-reactive protein (CRP), and rheumatoid factor (RF). Plain posterior-anterior radiographs of both hands were taken at the beginning of the study and 96 weeks later.

Table 1. Characteristics of patients with rheumatoid arthritis, according to study groups

	Methotrexate (n = 23)	Bucillamine (n = 24)	Combination (n = 24)
Female (%)	16 (69.6)	20 (83.3)	17 (70.8)
Age (years)	52.7 ± 9.3	52.5 ± 11.3	49.1 ± 12.9
Age range (years)	34–70	23–67	29–69
Height (cm)	157.9 ± 9.7	156.2 ± 7.2	158.8 ± 8.5
Weight (kg)	52.2 ± 9.0	53.3 ± 10.3	54.6 ± 8.9
Duration of illness (months)	8.2 ± 4.8	9.1 ± 5.3	10.6 ± 6.6
Steinbrocker stage (cases)			
Stage I	14	15	15
Stage II	9	9	9
Steinbrocker class (cases)			
Class I	4	2	3
Class II	16	18	19
Class III	3	4	2
Positive rheumatoid factor (%)	19 (82.6)	19 (79.2)	23 (95.8)
Corticosteroid therapy (%)	8 (34.8)	8 (33.3)	4 (16.7)
Dose (PSL eq) mg/day	4.6 ± 0.74	4.3 ± 1.4	4.8 ± 3.1
Previous DMARD (%)	4 (17.4)	9 (37.5)	4 (16.7)
No. of swollen joints	9.3 ± 5.9	11.5 ± 6.6	8.9 ± 4.3
No. of tender joints	15.9 ± 10.6	13.7 ± 8.3	13.6 ± 7.0
mHAQ	6.65 ± 3.72	5.79 ± 3.37	6.52 ± 3.70
Pain	67.0 ± 26.4	66.1 ± 26.3	61.5 ± 22.3
Global assessment[a]			
By patient	68.0 ± 21.8	66.7 ± 29.2	62.2 ± 22.0
By physician	69.8 ± 13.0	69.8 ± 22.8	65.6 ± 19.5
ESR (mm/h)	76.8 ± 39.3	71.7 ± 28.6	68.5 ± 33.7
CRP (mg/dl)	4.99 ± 4.31	4.13 ± 3.56	4.29 ± 4.53

PSL eq, prednisolone equivalent; DMARD, disease-modifying antirheumatic drug; mHAQ, Modified Health-Assessment Questionnaire; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein

[a] Global assessment of disease activity

HLA-DRB1 genotype was determined at the beginning of the study.

Therapeutic efficacy was clinically evaluated based on the ACR 20 and 50 responses.[15] The progress of joint destruction was evaluated using the difference in the total Sharp score at the commencement of the study and 96 weeks later. The total Sharp score, the sum of the erosion score, and the joint space narrowing score of the plain radiographs of the hands was the mean of the scores determined individually by three rheumatologists (Y.I., N.H., H.Y.) using a modified Sharp method.[16]

Fisher's exact test was used to test significance in contingency table analyses. The Kaplan–Meier cumulative survival curve maintaining ACR 20 response was also compared between the three treatment groups, whereas the statistical significance was determined by log-rank test.

Results

Seventy-one subjects were included in this study, and data were analyzed on an intention-to-treat basis. Table 1 shows the background factors of the 23 subjects in the MTX group, 24 subjects in the BUC group, and 24 subjects in the combination group. The mean disease duration was 8.2, 9.1, and 10.6 months, respectively. The majority of subjects were in Stage I of Steinbrocker's staging, and nearly 40% were in Stage II. Many subjects were in functional status Class 2.

Rheumatoid factor was positive in 82.6%, 79.2%, and 95.8% of subjects, respectively. The mean swollen joint counts were 9.3, 11.5, and 8.9, and painful joint counts 15.9, 13.7, and 13.6, respectively. The mean ESR for each group ranged from 68.5 to 76.8 mm/h, whereas the mean CRP ranged from 4.13 to 4.99 mg/dl. There were no statistically significant differences between the three groups for any of these indices.

Table 2 shows the ACR 20 and 50 response rates of the three treatment groups at the final examination for each subject. The ACR 20 response rate in the combination group was 79.2%, significantly higher than the rate of 43.5% for the MTX group (P = 0.008) and 45.8% of the BUC group (P = 0.0173). The ACR 50 response rate was 58.3% in the combination group, 34.8% in the MTX group, and 37.5% in the BUC group, the differences being not statistically significant. The study was interrupted due to insufficient response for two subjects (8.3%) in the combination group, significantly fewer than the 10 subjects (43.5%) in the MTX group and 10 subjects (41.7%) in the BUC group (P = 0.0171 and P = 0.0355, respectively).

Figure 1 shows the cumulative survival curve of the subjects maintaining the ACR 20 response rate in each group by the Kaplan–Meier method. The cumulative survival rate

Table 2. ACR 20 and ACR 50 responses and discontinuation due to lack of efficacy observed during treatment in three treatment groups

	Methotrexate	Bucillamine	Combination
No. of cases	23	24	24
ACR 20 response (%)	10 (43.5)[††]	11 (45.8)[†]	19 (79.2)
ACR 50 response (%)	8 (34.8)	9 (37.5)	14 (58.3)
Discontinued due to lack of efficacy (%)	10 (43.5)**	10 (41.7%)*	2 (8.3%)

[†] $P = 0.0355$, [††] $P = 0.0171$, * $P = 0.0173$, ** $P = 0.008$ vs combination

Table 3. Changes in total Sharp score over a 96-week period in patients treated for at least 24 weeks

	Methotrexate ($n = 15$)	Bucillamine ($n = 16$)	Combination ($n = 15$)
Total Sharp score at entry	24.3 ± 20.3	19.4 ± 14.4	14.4 ± 10.4
Changes in total Sharp score at 96 weeks	27.4 ± 31.2*	28.5 ± 26.2**	12.6 ± 9.0

* $P = 0.088$, ** $P = 0.034$ vs combination

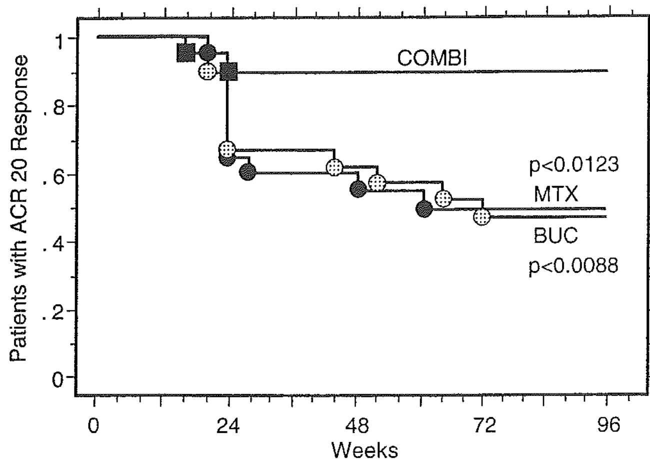

Fig. 1. Kaplan–Meier cumulative survival curve of the subjects maintaining the ACR 20 response rate. *Black circles*, methotrexate (*MTX*) group; *gray circles*, bucillamine (BUC) group; *squares*, combination (*COMBI*) group. The cumulative survival rate of the combination group was significantly higher than that of the MTX and BUC groups by log-rank test analysis ($P = 0.0123$ and $P = 0.0088$, respectively)

of the combination group was significantly higher than that in the MTX and BUC groups by log-rank test analysis ($P = 0.0123$ and $P = 0.0088$, respectively).

Table 3 shows the change in the total Sharp score over 96 weeks for subjects who continued the test drugs for more than 24 weeks. The increase in the total Sharp score during 96 weeks was 12.6 ± 9.0 in the combination group, lower than those of 27.4 ± 31.2 and 28.5 ± 26.2 in the MTX and BUC groups, respectively ($P = 0.088$ and $P = 0.034$), although there was no significant difference between the scores for each group at the beginning of the study. The increase in the total Sharp score in the combination group was also significantly lower than the value of 28.0 ± 28.3 for the single DMARD (combined MTX and BUC) group ($P = 0.0468$).

Treatment was discontinued due to adverse reactions in two subjects (8.7%) in the MTX group, six subjects (25.0%) in the BUC group, and seven subjects (29.2%) in the combination group, with no statistically significant differences between groups. The adverse reactions observed were as follows: one case each of leukopenia and proteinuria in the MTX group; three cases of rash, two of elevated transaminases, and one of proteinuria in the BUC group; and three cases of proteinuria and one each of elevated transaminases, rash, taste dyscrasia, and urinary tract infection in the combination group.

Discussion

Bucillamine is a sulfhydryl (SH) compound with a similar structure to D-penicillamine, but has two SH groups per molecule while D-penicillamine has one.[17] Both drugs suppress the proliferation and interleukin-2 production of T cells, although BUC is characteristically able to act without cupric ion,[18] and is currently widely used as an antrheumatic drug in Japan.

O'Dell et al. showed that simultaneous administration of MTX, SSZ, and HCQ is significantly more effective than MTX monotherapy or combination therapy with SSZ and HCQ.[12] They demonstrated an additive therapeutic effect for this combination of DMARDs. In addition, it has been reported that some combinations of biologic agents and a conventional DMARD, especially MTX, are more effective than treatment with an individual drug alone.[1,19]

On the other hand, there have been many reports of double-blind controlled studies investigating the usefulness of a combination of two conventional DMARDs, but none of these have shown statistically significant superiority for the combination compared to monotherapy.[2–7] In the present study, however, therapeutic efficacy was clearly shown to be higher in the combination group than in the

MTX or BUC groups, whereas the incidence of adverse reactions causing interruption of the study was not significantly different between the three groups.

The MTX dosage in this study was 8 mg/week, less than the dosages used in earlier studies.[12,20,21] The mean body weight of subjects in this study was, however, relatively light at 51.4 kg. The ACR 20 response rate at this dosage was 43.5%, consistent with reports of studies using MTX with[20] and without[21] folic acid. The ACR 20 response rate in the BUC group was 45.8%, similar to that in the MTX group, and 79.2% in the combination group, clearly higher than the other groups. The ACR 20 response rate in the combination group in this study was consistent with the 78% reported for the three DMARD combination therapy by O'Dell et al.[21]

Progress of bone destruction over the 96-week study period, as determined by Sharp's method modified by van der Heijde, was analyzed on an ITT (intention-to-treat) basis, giving a result of 31.7 ± 33.4 in the MTX group, 27.8 ± 31.0 in the BUC group, and 15.8 ± 15.8 in the combination group, with no statistically significant differences found between groups. Since the final dosage was reached at 13 weeks, it was considered appropriate to analyze all subjects administered test drugs for at least 24 weeks in order to compare the increases in the total Sharp score over 96 weeks between the three groups. The progress of bone destruction in the combination group was significantly lower than that of the BUC group, and showed a similar trend in comparison with the MTX group (Table 3). In addition, the increase in the Sharp score in the combination group was significantly lower than that in the combined MTX and BUC groups, i.e., the DMARD monotherapy group. These results suggest that combination therapy is superior to DMARD monotherapy not only in improvement of clinical symptoms, but also in suppression of bone destruction.

The present study demonstrated that the combination of MTX and BUC showed an additive clinical effect, using relatively small subject numbers, whereas earlier studies[2–7] had failed to show statistically significant superiority of combination therapies with various combinations of DMARDs. Bucillamine is known to suppress the function of T lymphocytes and B lymphocytes,[22] and inhibit the production of vascular endothelial growth factor (VEGF) in synovial cells.[23] On the other hand, MTX is known to suppress not only the function of lymphocytes[24,25] but also the function of neutrophils,[26] macrophages, and monocytes,[27] each of which has an important role in the pathogenesis of rheumatoid arthritis. The results of this study, showing increased therapeutic efficacy with combination therapy, suggest that these two DMARDs act on two or more different points of the pathogenetic process of rheumatoid arthritis.

Names of collaborating members and institutions: the MTX–BUC Combination Study Group, the Japanese Ministry of Health, Labour and Welfare's "Research for Establishment of Therapeutic Guidelines in Early Rheumatoid Arthritis".

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Single Dose of OCH Improves Mucosal T Helper Type 1/T Helper Type 2 Cytokine Balance and Prevents Experimental Colitis in the Presence of Vα14 Natural Killer T Cells in Mice

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Background and Aims: Vα14 natural killer T (NKT) cells seem to play important roles in the development of various autoimmune diseases. However, the pathophysiologic role of NKT cells in inflammatory bowel disease remains unclear. To clarify the mechanism by which the activation of NKT cells mediates protection against intestinal inflammation, we investigated the antiinflammatory role of specifically activated Vα14 NKT cells by glycolipids in a mouse experimental model of colitis induced by dextran sulfate sodium (DSS).

Methods: Colitis was induced in C57BL/6 mice by the oral administration of 1.5% DSS for 9 days. A single dose of OCH or α-galactosylceramide, a ligand for NKT cells, was administered on day 3 after the induction of colitis. Body weights and colonic mucosal injury were assessed in each glycolipid-treated group. Interferon-γ and interleukin-4 levels in the supernatants from colonic lamina propria lymphocytes (LPLs) were measured by enzyme-linked immunosorbent assay.

Results: The administration of a single dose of OCH attenuated colonic inflammation, as defined by body weights and histologic injury. The protective effects of OCH could not be observed in Vα14 NKT cell-deficient mice. In vivo treatment with OCH had improved the interferon-γ/interleukin-4 ratio from colonic LPLs on day 9 after DSS treatment.

Conclusion: The present data indicated that the activation of Vα14 NKT cells by OCH plays a pivotal role in mediating intestinal inflammation via altered mucosal T-helper type 1/type 2 responses. Therapeutic strategies that are designed to activate specifically Vα14 NKT cells may prove to be beneficial in treating intestinal inflammation.

Key Words: colitis, natural killer T cells, OCH, T helper type 1/T helper type 2

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Natural killer T (NKT) cells have been identified as a novel lymphoid lineage distinct from conventional T cells and natural killer (NK) cells. NKT cells express both invariant Vα14 NKT-specific antigen receptor as well as an NK marker (NK1.1).[1–5] The specific features of this cell type include a limited repertoire with an invariant Vα chain consisting of the Vα14-Jα281 gene segment and the highly skewed Vβ chains Vβ8.2, Vβ7, and Vβ2 in mice. NKT cells are restricted by the nonclassical major histocompatibility complex class I-like molecule CD1d, which is expressed on cells of hematopoietic origin as well as on intestinal epithelial cells.[6–10] These cells recognize glycolipid antigens such as α-galactosylceramide (αGalCer), a glycolipid that is isolated from marine sponges that specifically binds CD1d.[11–14] NKT cells are abundant in the thymus, liver, and bone marrow, and are also found in peripheral lymphoid organs. It has been reported that NKT cells play an important role in various aspects of the immune response, including the regulation of allergic and autoimmune diseases[15–18] and the prevention of tumor metastasis.[19–22]

One of the mechanisms by which NKT cells elicit the effector function is through the production of large amounts of interferon (IFN)-γ, interleukin (IL)-4, and IL-10 in response to various stimuli.[1] In vitro and in vivo studies have shown that the cytokine profiles of these cells depend both on the nature of the activating stimulus and on the nature of the cytokines, and on other soluble factors in the local microenvironment. The activation of $NK1.1^+$T cells by CD3 cross-linking or CD1 results in the production of both IFN-γ and IL-4, whereas the stimulation of $NK1.1^+$ results in the production of IFN-γ

only.[13,23,24] IL-12 stimulates NK1.1^{+}T cells to produce IFN-γ and inhibits their production of IL-4,[23,25,26] whereas IL-4 production by these cells requires IL-7 and is promoted by glucocorticoids.[27–29] Several costimulatory molecules play a role in the regulation of these cytokines. In the presence of blocking B7.2 (CD86) monoclonal antibody, αGalCer stimulation shifts the cytokine profile of NKT cells toward T helper (T_H) type 2 cells, whereas the presentation of αGalCer by CD40-activated antigen presenting cells causes a T_H1 shift of NKT cells.[30] Recently, Miyamoto et al[31] demonstrated that OCH, which has shorter hydrophobic chains than αGalCer, induces the production of, predominantly, IL-4 by NKT cells from murine spleen, leading to T_H2 bias in a Vα14 NKT cell-dependent manner. Therefore, the specific stimulation of NKT cells may have a therapeutic effect on various diseases associated with the T_H1-type or T_H2-type immune response.

The role of NKT cells in intestinal inflammation has been elucidated by several investigators. The ligand-specific activation of Vα14 NKT cells by αGalCer has been shown to protect mice against experimental colitis.[32] This protection was absent in CD1d$^{-/-}$ mice, and the elimination of NK1.1^{+} cells reduced the effect of αGalCer. Other authors have reported that oxazolone-induced colitis, a T_H2-type colitis, is mediated by IL-13–producing NKT cells.[33] These results suggest that a CD1d–NKT cell interaction may be involved in the pathogenesis of colonic inflammation. However, the precise mechanism by which activated NKT cells modulate the pathogenesis of colitis is not yet understood. In the present study, we examined the role of NKT cells activated by OCH or αGalCer in protection against dextran sulfate sodium (DSS)-induced colitis. Our results indicate that the activation of Vα14 NKT cells by OCH shifted toward T_H2-type immune balance in the intestinal mucosa and that this is critical for protection against DSS-induced colitis.

MATERIALS AND METHODS

Mice

Specific pathogen-free C57BL/6 (B6) mice were purchased from Japan Clea (Tokyo, Japan). Jα281-deficient (Vα14 NKT cell-deficient [KO]) mice on a B6 background were generated, as described previously.[20] All mice were housed under specific pathogen-free conditions in microisolator cages in the animal facility at Hiroshima University, and only male mice (9 to 11 wk of age) were used.

DSS Colitis Model

DSS ([molecular weight, 5000] Wako Chemical Co, Osaka, Japan) was added to the water supply of the animals at a concentration of 1.5% (wt/vol) for days 1–9. The progression of colitis was monitored by a daily examination for rectal bleeding, perianal soiling, lack of grooming, hunched posture, weight loss, and mortality. Total body weight (in grams) was measured at the same time each day. All experiments were repeated at least twice with 5 to 15 mice.

In Vivo Injection of Glycolipid

OCH and αGalCer were first dissolved in dimethylsulfoxide at 100 μg/mL and then were diluted in phosphate-buffered saline (PBS) solution. To investigate the role of invariant NKT cells on the induction phase of DSS-induced colitis, each glycolipid (100 μg/kg in 200 μL of solution) was injected intraperitoneally on day 3 after the induction of colitis. Day 3 was selected because, in our preliminary studies, a single dose of glycolipids before the administration of DSS did not show any protective effect against DSS-induced colitis. Control animals received 200 μL of PBS solution containing the same concentration of dimethylsulfoxide (10%).

Assessment of the Severity of Colitis

Mice were killed on day 9 after DSS administration. Intestinal tissues were removed and opened longitudinally. The length of the colon was measured after the exclusion of the cecum and prior to division for histology. The tissues then were rolled concentrically and embedded in paraffin. Sections were stained with hematoxylin-eosin. The degree of inflammation of the colon was graded for severity according to mucosal damage (D) and the extension of the lesion (E) based on the method of Kitajima et al.[34] The histologic index was calculated as D plus E and was expressed as the mean of the score for each segment (i.e., for the cecum and the proximal, middle, and distal colon). The total score was the sum of the scores obtained in these sections. All slides were scored blindly.

Isolation of Colonic Lamina Propria Lymphocytes

Colonic lamina propria lymphocytes (LPLs) were isolated as described previously.[35] Briefly, nonadherent mesenteric tissues were removed, and the entire length of the intestine was opened longitudinally, washed with PBS solution, and cut into small (~5-mm) pieces. The dissected mucosa was incubated with $Ca^{++}Mg^{++}$-free Hanks balanced salt solution containing 1 mM ethylenediaminetetraacetic acid (Sigma, St. Louis, MO) for 20 minutes. Specimens were washed with Hanks balanced salt solution and then were incubated in 150 U/mL collagenase (Wako Chemical Co) in RPMI 1640 medium for 1.5 hours at 37 °C with stirring. Cells were suspended in 44% isotonic Percoll (Sigma) underlaid with 66% isotonic Percoll and were centrifuged for 20 minutes at 2200 revolutions per minute at room temperature. Cells at the interface were collected and washed twice with cold PBS. Approximately 2×10^6 cells per colon were recovered with >95% viability, as determined by trypan blue exclusion. Cells not excluding trypan blue were not included in the final count.

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Cytokine Analysis in the Colonic Mucosa

Colonic LPLs were purified, transferred to 96-well plates (5 × 10^5 cells per well), and cultured for 48 hours in medium containing 500 ng/mL phorbol myristate acetate (PMA) (Sigma) and 50 ng/mL ionomycin (Sigma). After 48 hours, supernatants were harvested and stored at –20 °C until further analysis. The colon organ culture analysis for cytokines was performed as described previously.[36] Briefly, the mice were killed, the colon was removed, cut open longitudinally, and washed in PBS solution. The colonic tissue was transferred to 24-well flat-bottom culture plates containing fresh RPMI 1640 medium supplemented with penicillin and streptomycin, and was incubated at 37 °C for 24 hours. Culture supernatants were harvested and assayed for cytokines. IFN-γ, IL-4, and IL-10 were measured with OptEIA kits (BD, San Jose, CA). All samples were analyzed in triplicate.

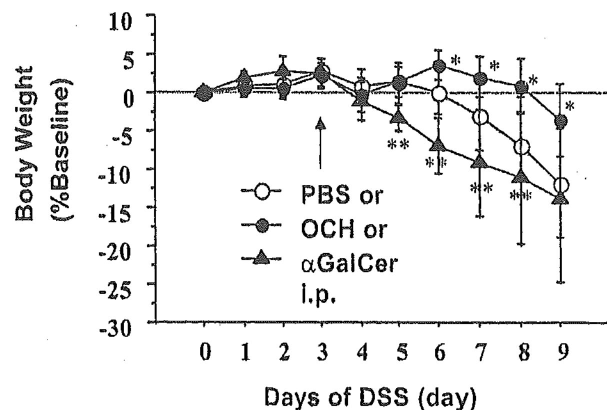
Statistical Analysis

Data were analyzed with the Japanese version of StatView software (Hulinks, Tokyo, Japan) on a Macintosh Computer (Apple Computer, Cupertino, CA). The data are expressed as the mean ± SD. Differences between groups were examined for statistical significance with the Student *t* test after analysis of variance. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Efficacy of In Vivo Glycolipid Treatment in DSS-Induced Colitis

As reported previously, OCH, a relatively new synthetic analogue of αGalCer, induces the production of IL-4 by NKT cells from murine spleen, leading to T_H2 bias in a Vα14 NKT cell-dependent manner.[31] To investigate whether the specific activation of Vα14 NKT cells by OCH protects against colitis, a single dose of OCH was administered to C57BL/6 mice by intraperitoneal injection on day 3 during the induction of colitis. As shown in Figure 1A, OCH-treated mice lost significantly less weight compared with PBS-treated mice. Gross rectal bleeding was seen in 60% of PBS-treated mice (6/10 mice) and in 10% of OCH-treated mice (1/10 mice) on day 9. OCH significantly prevented shortening of the colon (Fig. 1B). Histologic analysis confirmed the presence of marked inflammatory cell infiltrations with a loss of the mucosal surface in the colons of mice injected with PBS (Figs. 2A, D). In contrast, mononuclear cell infiltration was observed, but colonic crypts were still conserved in the colons of OCH-treated mice (Figs. 2B, E). The histologic scores of the severity of colitis were significantly reduced in the OCH-treated group (Figs. 3A, B). PBS-treated and OCH-treated mice began to lose their initial body weight on day 6 and day 8, respectively (Fig. 1A), and all OCH-treated animals had histologic colitis on day 9. OCH may therefore delay colitis by 2 days rather than provide complete protection from colitis.



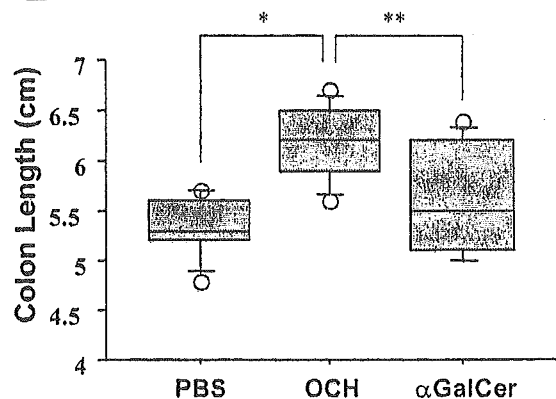


FIGURE 1. The effect of OCH on the protective immunity against DSS-induced colitis. A, C57BL/6 mice (n = 10 per group) were exposed continuously to 1.5% DSS in drinking water from day 0 to day 9. Mice were injected intraperitoneally with 100 μg/kg OCH, αGalCer, or PBS solution on day 3. Body weights of individual mice were recorded daily. The measurement of body weight, as a percentage of starting weight, is shown. * = $P < 0.005$ compared with mice treated with PBS; ** = $P < 0.05$ compared with mice treated with PBS. B, Comparison of colon lengths in DSS-treated mice on day 9. Each box plot represents 10 mice. * = $P < 0.0001$ for a comparison of OCH versus PBS; ** = $P < 0.05$ for a comparison of OCH versus αGalCer.

A single dose of αGalCer also improved the histologic score in the middle and proximal parts of the colon at the same levels as OCH treatment (Figs. 2 and 3A). When the scores were pooled with differences in the other sites, OCH was superior to αGalCer in histology (Fig. 3B). Gross rectal bleeding was observed in 50% of αGalCer-treated mice (5/10 mice) on day 9. In total, αGalCer treatment resulted in no differences in body weight changes, colon length, or total histologic scores in comparison with PBS treatment (Figs. 1–3). These data demonstrated that OCH is more effective in preventing DSS-

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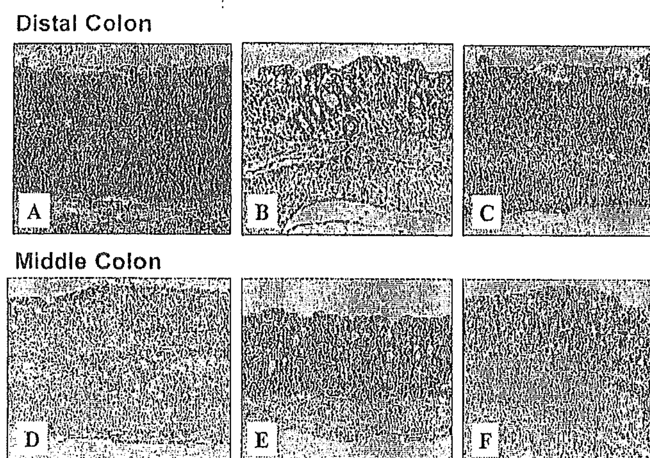

FIGURE 2. Histopathology of the colons from DSS-treated C57BL/6 mice. Representative photomicrographs (200×) of paraffin-embedded, hematoxylin-eosin–stained longitudinal sections of the distal parts of the colon (A–C) and the middle parts of the colon (D–F) from mice injected intraperitoneally with PBS solution (A and D), OCH (B and E), and αGalCer (C and F).

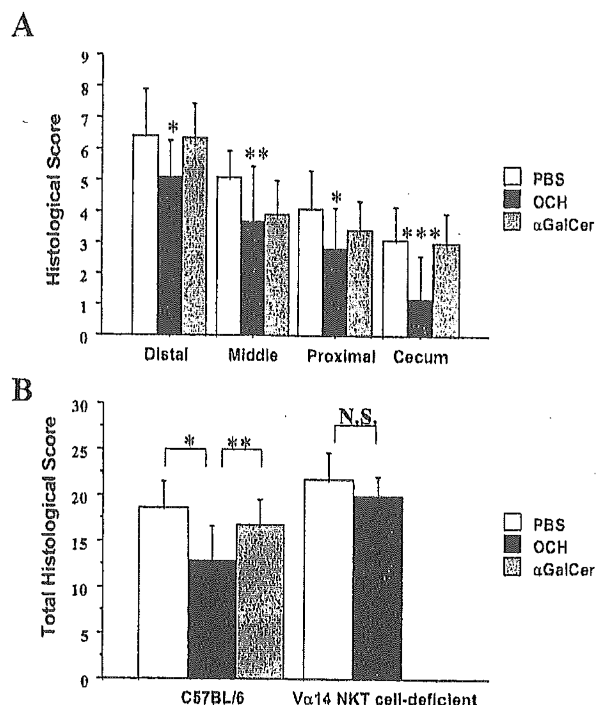

FIGURE 3. Histologic scores. Scoring was performed as described in "Materials and Methods." A, Histologic scores of each part of the colons. * = $P < 0.05$ for a comparison of PBS versus OCH; ** = $P < 0.05$ for a comparison of PBS versus OCH or αGalCer; and *** = $P < 0.005$ for a comparison of PBS versus OCH. B, Total histologic scores were expressed as the sum of the scores obtained in these sections. Vα14 NKT cell-deficient mice (n = 10 per group) also were treated with 1.5% DSS in drinking water from day 0 to day 9. The mice were injected intraperitoneally with 100 μg/kg OCH or PBS solution on day 3. * = $P < 0.005$ for a comparison of PBS versus OCH; ** = $P < 0.05$ for a comparison of OCH versus αGalCer in C57BL/6 mice; N.S. = not significant.

induced colitis than αGalCer. To confirm whether this protective effect of OCH was Vα14 NKT cell-dependent, we administered OCH to Vα14 NKT cell-deficient mice on day 3 during the induction of colitis. As shown in Figure 3B, OCH treatment had no effect on prevention of the development of DSS-induced intestinal inflammation in Vα14 NKT cell-deficient mice, as determined by evaluation of the total histologic score. These data indicated that the activation of Vα14 NKT cells by specific glycolipids influences protective immunity against intestinal inflammation.

Effect of Glycolipids on Mucosal Cytokine Balance

To examine whether the specific activation of Vα14 NKT cells by in vivo glycolipids could regulate the mucosal T_H1/T_H2 balance, we measured IFN-γ and IL-4 levels in supernatants from in vitro-stimulated colonic LPLs by enzyme-linked immunosorbent assay (ELISA) after the intraperitoneal injection of glycolipids. Colonic LPLs from DSS-treated C57BL/6 mice on days 5 and 9 produced significantly higher levels of both IFN-γ and IL-4 in comparison with those from non–DSS-treated, control, day 0 animals (Fig. 4A). The IFN-γ/IL-4 ratio increased in a time-dependent manner, suggesting that the progression of intestinal inflammation may be associated with T_H1-predominant immune responses (Fig. 4B). We next studied whether this ratio was affected by the administration of glycolipids. Treatment with both glycolipids induced higher amounts of IFN-γ and IL-4 than did PBS treatment (Fig. 4C, upper panels). When the IFN-γ/IL-4 ratio was calculated in each supernatant, both glycolipids significantly improved the ratio, and the degree of improvement by OCH was greater than that by αGalCer (Fig. 4C, lower panel). IL-10 had an anti-inflammatory effect on DSS-induced colitis.[37] We then analyzed IL-10 levels in the supernatants of colon organ cultures at an early phase after the injection of the glycolipids. Interestingly, OCH injection induced significantly higher IL-10 production than did αGalCer in the local colonic mucosa at 6 and 12 hours after injection (Figs. 5A, B). This IL-10 production was abrogated in Vα14 NKT cell-deficient mice, suggesting that the colonic mucosal IL-10 production is Vα14 NKT cell-dependent (data not shown). These data indicate that OCH induces a sufficient production of IL-10 in the local colonic mucosa and improves the subsequent mucosal T_H1/T_H2 cytokine balance at the time of development of colitis.

DISCUSSION

Here, we have shown that the specific activation of Vα14 NKT cells by OCH protects against DSS-induced colitis

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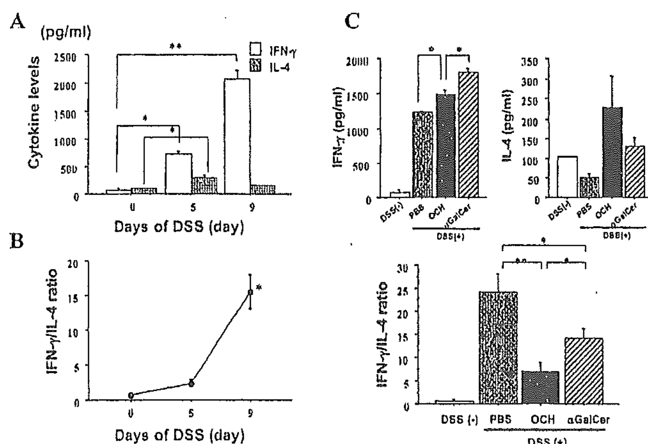

FIGURE 4. Comparison of the effects of glycolipids on mucosal cytokine balance in in vitro-stimulated colonic LPLs. A, C57BL/6 mice (n = 5 per group) were killed on the indicated days after 1.5% DSS administration, and colonic LPLs were purified. LPLs were stimulated in vitro with 500 ng/mL PMA and 50 ng/mL ionomycin, and were incubated at 37 °C for 48 hours. Supernatants were harvested, and cytokine levels were assessed by enzyme-linked immunosorbent assay. * = $P < 0.05$ for a comparison of day 0 versus day 5; ** = $P < 0.005$ for a comparison of day 0 versus day 9. B, Levels of IFN-γ and IL-4 were determined, and the IFN-γ/IL-4 ratio was calculated. * = $P < 0.005$ versus day 0. C, C57BL/6 mice treated with 1.5% DSS were injected with 100 μg/kg OCH, αGalCer, or PBS solution on day 3. The mice were killed on day 9, and colonic LPLs were purified. The LPLs were stimulated in vitro with 500 ng/mL PMA and 50 ng/mL ionomycin, and were incubated at 37 °C for 48 hours. Supernatants were harvested, and cytokine levels were analyzed by enzyme-linked immunosorbent assay. The amounts of IFN-γ and IL-4 were determined, and the IFN-γ/IL-4 ratio was calculated. DSS (−) and DSS (+) represent non-DSS-treated and DSS-treated mice, respectively. * = $P < 0.05$ for a comparison of OCH versus PBS or αGalCer, or PBS versus αGalCer. ** = $P < 0.01$ for a comparison of OCH versus PBS. Bars indicate the mean ± SD of 5 mice per group. The data are representative of 3 independent experiments.

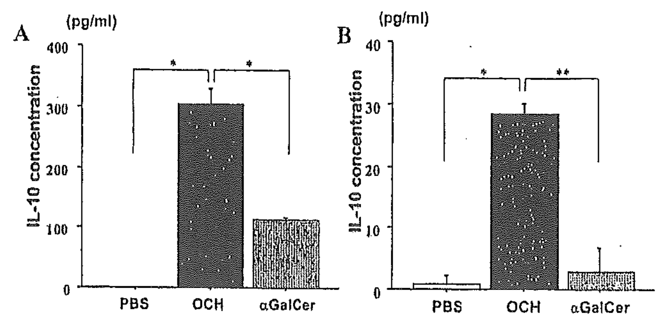

FIGURE 5. Rapid IL-10 induction after in vivo glycolipid injection during DSS administration. C57BL/6 mice treated with 1.5% DSS were injected with 100 μg/kg OCH, αGalCer, or PBS solution on day 3. The mice were killed 6 hours after injection (A) and 12 hours after injection (B), the colons were harvested, and organ culture was performed for cytokine analysis. The data are representative of 2 independent experiments. Bars indicate the mean ± SD of 5 mice per group. * = $P < 0.005$ for a comparison of OCH versus PBS or αGalCer; ** = $P < 0.05$ for a comparison of OCH versus αGalCer.

through the modulation of the mucosal T_H1/T_H2 cytokine balance.

It was recently reported that an analog of αGalCer, OCH, which has a truncated sphingosine chain, stimulates NKT cells to produce IL-4.[31] Therefore, OCH has the potential to elicit protective immunity against T_H1-mediated inflammatory disease. We have shown that a single dose of OCH attenuates DSS-induced colitis. This protection was mediated by Vα14 NKT cells because OCH did not elicit any protective effect in Vα14 NKT cell-deficient mice. In our preliminary study, we compared Vα14 NKT cell-deficient mice to wild-type B6 mice by administering 1.5% DSS. Interestingly, the knockout mice showed more severe intestinal inflammation when treated with DSS (our unpublished data). The loss of the protective effect of OCH in Vα14 NKT cell-deficient mice, however, may not be due to the increased susceptibility to DSS colitis because the effect of OCH was not observed when the knockout mice were given a lower dose (1.0%) of DSS (data not shown). These findings suggest that the specific activation of Vα14 NKT cells could reduce intestinal inflammation.

In contrast, a single dose of αGalCer, which was originally discovered as a ligand for NKT cells, had a smaller effect on prevention against DSS-induced colitis than OCH. Why do these glycolipids differ in the ability to protect against intestinal inflammation?

To examine the effects of these glycolipids on local immunologic responses, the levels of cytokines produced by colonic LPLs were analyzed. Colonic LPLs from DSS-treated C57BL/6 mice produced a significantly higher IFN-γ/IL-4 ratio in comparison to that from non–DSS-treated control animals. This ratio increased in a time-dependent fashion. We found in the present study that this ratio was significantly decreased by treatment with glycolipids, and OCH improved the ratio more significantly than αGalCer. The severity of the disease inversely correlated with the IFN-γ/IL-4 ratio. Therefore, OCH may prevent colitis through improvement of the mucosal T_H1/T_H2 cytokine balance.

The T_H1/T_H2 response in DSS-induced colitis remains unclear. It has been reported that DSS-induced colitis in C57BL/6 mice is characterized by a T_H1-type response with a strong induction of IFN-γ messenger RNA expression.[38] In DSS-treated mice, anti–IFN-γ and/or anti-tumor necrosis factor-α antibodies significantly reduce the severity of colitis.[39] On the other hand, the role of IL-4 in DSS-induced colitis is not fully understood. Stevceva et al[40] showed that DSS-induced colitis is ameliorated in IL-4–deficient mice, suggesting that even IL-4 may play a pathologic role in the intestinal inflammation induced by DSS. Therefore, the beneficial effects of the

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glycolipids in the DSS-induced colitis model may not be due simply to an increase in IL-4 production.

Another T_H2-related cytokine, IL-10, has been widely characterized as an immunosuppressive cytokine and is important for mucosal immunologic homeostasis.[41–43] We also detected that OCH rapidly induces the localized expression of IL-10 in the colonic mucosa. How does OCH induce secretion of IL-10? Although we could not determine which cells in the colonic mucosa produce IL-10 in response to the activation of NKT cells by OCH, dendritic cells are one of the main producers of IL-10,[44] and CD1d-restricted NKT cells are known to contribute to immune function by regulating dendritic cell maturation.[45] A recent study showed that αGalCer stimulates the production of IL-12 by dendritic cells.[46] OCH has shorter hydrophobic chains than does αGalCer but has the same hydrophilic cap.[31] Therefore, OCH may bind to the CD1d groove less stably and may induce a weaker T-cell receptor signal to CD1d-restricted T cells than does αGalCer. Such T cells could induce antiinflammatory mature dendritic cells that produce more IL-10 than IL-12.[47] We hypothesized that the difference in the affinities of the glycolipids for the CD1d groove may influence the development of mature dendritic cells that preferentially produce IL-12 or IL-10. Since the murine colonic mucosa contains dendritic cells, which have a capacity to produce both IL-10 and IL-12,[48] it would be important to know whether these dendritic cell-derived cytokine balances are affected by activating NKT cells with glycolipids. Further studies are needed to clarify the precise mechanism underlying the protective immunity induced when NKT cells are activated.

It was recently shown that a course of multiple injections of αGalCer provides some protection against DSS-induced colitis.[32] Our present findings indicated that a single injection of αGalCer is not sufficient to elicit a preventive effect against DSS-induced colitis. This difference may be explained by the fact that single and multiple injections of αGalCer are known to induce specifically T_H1-predominant[49,50] and T_H2-predominant[51–53] immune responses, respectively. Miyamoto et al[31] reported that a single injection of OCH, but not of αGalCer, improves experimental allergic encephalomyelitis, a T_H1-associated disease, in mice. These previous findings and the results of the present study suggest that shifting toward a T_H2-type mucosal immune response may be crucial for protecting against DSS-induced colitis.

Heller et al[33] showed that oxazolone-induced colitis, a T_H2 colitis model, is mediated by IL-13–producing NKT cells. Whether NKT cells act as effector cells or regulatory cells may depend on the pathophysiology of the disease. Recently, Fuss et al[54] demonstrated the presence of IL-13–producing nonclassical NKT cells in the colonic mucosa of patients with ulcerative colitis. It would be interesting to examine the interaction between these pathogenic noninvariant mucosal NKT cells and anti-inflammatory invariant NKT cells. According to our present study, OCH may have potential as a treatment of human T_H1-predominant intestinal inflammatory diseases, such as Crohn's disease.[55]

In summary, we showed that Vα14 NKT cells are important for attenuating DSS-induced colitis. This protective immunity may be modulated by the activation status of Vα14 NKT cells. Although additional experiments are needed, our data indicate that an early and sufficient T_H2-biased immune response in the intestinal mucosa during the onset of colitis may have an antiinflammatory effect. Future studies with Vα14 NKT cell-deficient mice and analyses of the effects of glycolipids in other animal models of colitis will clarify our understanding of the pathologic process underlying colitis and will improve the chances of developing effective treatments for human inflammatory bowel disease.

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Stimulation of Host NKT Cells by Synthetic Glycolipid Regulates Acute Graft-versus-Host Disease by Inducing Th2 Polarization of Donor T Cells[1]

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NKT cells are a unique immunoregulatory T cell population that produces large amounts of cytokines. We have investigated whether stimulation of host NKT cells could modulate acute graft-vs-host disease (GVHD) in mice. Injection of the synthetic NKT cell ligand α-galactosylceramide (α-GalCer) to recipient mice on day 0 following allogeneic bone marrow transplantation promoted Th2 polarization of donor T cells and a dramatic reduction of serum TNF-α, a critical mediator of GVHD. A single injection of α-GalCer to recipient mice significantly reduced morbidity and mortality of GVHD. However, the same treatment was unable to confer protection against GVHD in NKT cell-deficient CD1d knockout ($CD1d^{-/-}$) or $IL\text{-}4^{-/-}$ recipient mice or when $STAT6^{-/-}$ mice were used as donors, indicating the critical role of host NKT cells, host production of IL-4, and Th2 cytokine responses mediated by donor T cells on the protective effects of α-GalCer against GVHD. Thus, stimulation of host NKT cells through administration of NKT ligand can regulate acute GVHD by inducing Th2 polarization of donor T cells via STAT6-dependent mechanisms and might represent a novel strategy for prevention of acute GVHD. ***The Journal of Immunology*, 2005, 174: 551–556.**

Allogeneic hemopoietic stem cell transplantation (HSCT)[3] cures various hematologic malignant tumors, bone marrow (BM) failures, and congenital metabolic disorders. Emerging evidence suggests that allogeneic HSCT is also useful for treatment of other diseases, including solid tumors and autoimmune diseases (1, 2). However, graft-vs-host disease (GVHD) is a major obstacle that precludes wider application of allogeneic HSCT. The pathophysiology of acute GVHD is complex, involving 1) donor T cell responses to the host alloantigens expressed by host APCs activated by conditioning regimens (i.e., irradiation and/or chemotherapy), and 2) dysregulation of inflammatory cytokine cascades, leading to further T cell expansion and induction of cytotoxic T cell responses (3).

$CD4^+$ helper T cells can be divided into two distinct subpopulations: Th1 and Th2 cells (4). Th1 cells produce IFN-γ and IL-2, whereas Th2 cells produce IL-4, IL-5, and IL-13. Although the role of Th1 and Th2 cytokines in the pathophysiology of acute GVHD is complex and controversial (5–8). Th1 polarization of donor T cells predominantly plays a role in inducing the "cytokine storm" that is seen in several models of acute GVHD (3, 9), whereas Th2 polarization mostly suppresses inflammatory cascades and reduces acute GVHD (10–12). Many properties of dendritic cells (DCs), including the type of signal, the duration of activation, the ratio of DCs to T cells, and the DC subset that presents the Ag, influence the differentiation of naive $CD4^+$ T cells into Th1 or Th2 cells (13). The cytokines that are present during the initiation of the immune responses at the time when the TCR engages with MHC/peptide Ags are critically important for Th cell differentiation (14).

NKT cells are a distinct subset of lymphocytes characterized by expression of surface markers of NK cells together with a TCR. Although the NKT cell population exhibits considerable heterogeneity with regard to phenotypic characteristics and functions (15), the major subset of murine NKT cells expresses a semi-invariant TCR, $V\alpha 14\text{-}J\alpha 18$, in combination with a highly skewed set of Vβs, mainly Vβ8 (16). NKT cells can be activated via their TCR by glycolipid Ags presented by the nonpolymorphic MHC class I-like molecule CD1d expressed by APCs (17). Stimulation of NKT cells rapidly induces secretion of large amounts of IFN-γ and IL-4, thereby influencing the Th1/Th2 balance of conventional $CD4^+$ T cell responses (18). In particular, NKT cells are considered an important early source of IL-4 for the initiation of Th2 responses (19, 20), although these cells are not absolutely required for the induction of Th2 responses (21–23). NKT cells are absent in CD1d knockout ($CD1d^{-/-}$) mice because of defects in their thymic positive selection, which requires CD1d expression on hemopoietic cells, probably double-positive thymocytes (24, 25).

Considering the critical role of cytokines in the development of acute GVHD, we investigated the role of host NKT cells in an experimental model of GVHD, using synthetic NKT cell ligands,

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[3] Abbreviations used in this paper: HSCT, hemopoietic stem cell transplantation; BM, bone marrow; GVHD, graft-vs-host disease; DC, dendritic cell; α-GalCer, α-galactosylceramide; BMT, bone marrow transplantation; TBI, total body irradiation; TCD, T cell depletion; LN, lymph node; WT, wild type.

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α-galactosylceramide (α-GalCer) (26), a glycolipid originally purified from a marine sponge, and its analog, OCH (27). Our findings indicate that stimulation of host NKT cells with NKT ligands can modulate acute GVHD.

Materials and Methods

Mice

Female C57BL/6 (B6, H-2^b) and BALB/c (H-2^d) mice were purchased from Charles River Japan. IL-$4^{-/-}$ B6 and STAT$6^{-/-}$ BALB/c mice were purchased from The Jackson Laboratory. CD1d$^{-/-}$ B6 mice were established by specific deletion of the CD1d1 gene segment (22). Mice, between 8 and 16 wk of age, were maintained in a specific pathogen-free environment and received normal chow and hyperchlorinated drinking water for the first 3 wk post-bone marrow transplantation (BMT). All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research Center.

Bone marrow transplantation

Mice were transplanted according to a standard protocol described previously (28). Briefly, B6 mice received lethal total body irradiation (TBI; x-ray), split into two doses separated by 6.5 h to minimize gastrointestinal toxicity. Recipient mice were injected with 5×10^6 BM cells plus 5×10^6 spleen cells from either syngeneic (B6) or allogeneic (BALB/c) donors. T cell depletion (TCD) of donor BM cells was performed using anti-CD90 MicroBeads and the AutoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. Donor cells were resuspended in 0.25 ml of HBSS (Invitrogen Life Technologies) and injected i.v. into recipients on day 0. Survival was monitored daily. The degree of systemic acute GVHD was assessed weekly by a scoring system incorporating five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity, as described (29).

Glycolipids

α-GalCer, (2S,3S,4R)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000), was synthesized and provided by Kirin Brewery Company (30). A homologue of α-GalCer, OCH, was selected from a panel of synthesized α-GalCer analogues by replacing the sugar moiety and/or truncating the aliphatic chains, because of its ability to stimulate enhanced IL-4 and reduced IFN-γ production by NKT cells, as previously described (27, 31). BMT recipient mice were injected i.p. with α-GalCer or OCH (100 μg/kg) immediately after BMT on day 0. Mice from the control groups received the diluent only.

Flow cytometric analysis

mAbs used were FITC- or PE-conjugated anti-mouse CD4, H-$2K^b$, and H-$2K^d$ (BD Pharmingen). Cells were preincubated with 2.4G2 mAb (rat anti-mouse FcγR) for 10 min at 4°C to block nonspecific binding of labeled Abs, and then were incubated with the relevant mAbs for 15 min on ice. Finally, cells were washed twice with 0.2% BSA in PBS and fixed. After lysis of RBCs with FACS lysing solution (BD Pharmingen), cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). 7-Amino-actinomycin D (BD Pharmingen)-positive cells (i.e., dead cells) were excluded from the analysis. Fluorochrome-conjugated irrelevant IgG were used as negative controls. At least 5000 live events were acquired for analysis.

Cell cultures

Mesenteric lymph nodes (LNs) and spleens were removed from animals 6 days after BMT and four to six mesenteric LNs or spleens from each experimental group were combined. Numbers of cells were normalized for T cells and were cultured in complete DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.02 mM 2-ME, and 10 mM HEPES in wells of a 96-well flat-bottom plate, at a concentration of 5×10^4 T cells/well with 1×10^5 irradiated (20 Gy) peritoneal cells harvested from naive B6 (allogeneic) animals, or with 5 μg/ml plate-bound anti-CD3ε mAbs (BD Pharmingen) and 2 μg/ml anti-CD28 mAbs (BD Pharmingen). Forty-eight hours after the initiation of culture, supernatants were collected for the measurement of cytokine levels.

ELISA

ELISA was performed according to the manufacturer's protocols (R&D Systems) for measurement of IFN-γ, IL-4, and TNF-α levels, as described previously (32). Samples were obtained from culture supernatant and blood from retro-orbital plexus, diluted appropriately, and run in duplicate. Plates were read at 450 nm using a microplate reader (Bio-Rad). The sensitivity of the assays was 31.25 pg/ml for IFN-γ, 7.6 pg/ml for IL-4, and 23.4 pg/ml for TNF-α.

Histology

Formalin-preserved livers and small and large bowels were embedded in paraffin, cut into 5-μm-thick sections, and stained with H&E for histological examination. Slides were coded without reference to prior treatment and examined in a blinded fashion by a pathologist (C. Liu). A semiquantitative scoring system was used to assess the following abnormalities known to be associated with GVHD, as previously described (33): 0, normal; 0.5, focal and rare; 1.0, focal and mild; 2.0, diffuse and mild; 3.0, diffuse and moderate; and 4.0, diffuse and severe. Scores were added to provide a total score for each specimen. After scoring, the codes were broken and data were compiled. Pathological GVHD scores of intestine are the sum of scores for small bowel and colon.

Statistical analysis

Mann-Whitney *U* test was applied for the analysis of cytokine data and clinical scores. We used the Kaplan-Meier product limit method to obtain survival probability, and the log-rank test was applied for comparing survival curves. Differences in pathological scores between the α-GalCer-treated group and the diluent-treated group were examined by two-way ANOVA. We defined $p < 0.05$ as statistically significant.

Results

Administration of α-GalCer stimulates lethally irradiated mice to produce IFN-γ and IL-4

We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. B6 mice were given 13 Gy TBI and were injected i.p. with α-GalCer, OCH, or diluent 2 h after TBI. Six hours later, blood samples were obtained, and serum samples were prepared for measurement of IFN-γ and IL-4. TBI alone or BMT itself did not stimulate diluent-treated mice to produce IFN-γ or IL-4 (Fig. 1). Administration of α-GalCer increased serum levels of IFN-γ and IL-4, even in mice receiving TBI. However, serum levels of IFN-γ were much less in irradiated mice than in unirradiated mice. By contrast, the ability of irradiated mice to produce IL-4 to α-GalCer was maintained for 48 h after irradiation. Serum levels of IFN-γ and IL-4 in response to α-GalCer were not altered when irradiated wild-type (WT) mice were injected with 5×10^6 BM cells and 5×10^6 spleen cells isolated from allogeneic (BALB/c) donors. Furthermore, these cytokine responses were not observed when α-GalCer was injected into irradiated NKT cell-deficient CD1d$^{-/-}$ mice with or without BMT. These results suggest that host NKT cells that survive for at least 48 h after irradiation, rather than from infused donor cells, are critically involved in the production of these cytokines in response to glycolipids. Irradiation appears to impair the ability of mice to produce IFN-γ while preserving IL-4 production in response to α-GalCer. Similar cytokine profiles were observed when OCH was administered (data not shown).

Administration of α-GalCer to recipients polarizes donor T cells toward Th2 cytokine production after allogeneic BMT

Induction of GVHD fundamentally depends upon donor T cell responses to host alloantigens. We next evaluated the effect of glycolipid administration on donor T cell responses after allogeneic BMT in a well-characterized murine model of acute GVHD (BALB/c→ B6) directed against both MHC and multiple minor histocompatibility Ags. Lethally irradiated B6 mice were transplanted with 5×10^6 BM cells and 5×10^6 spleen cells from

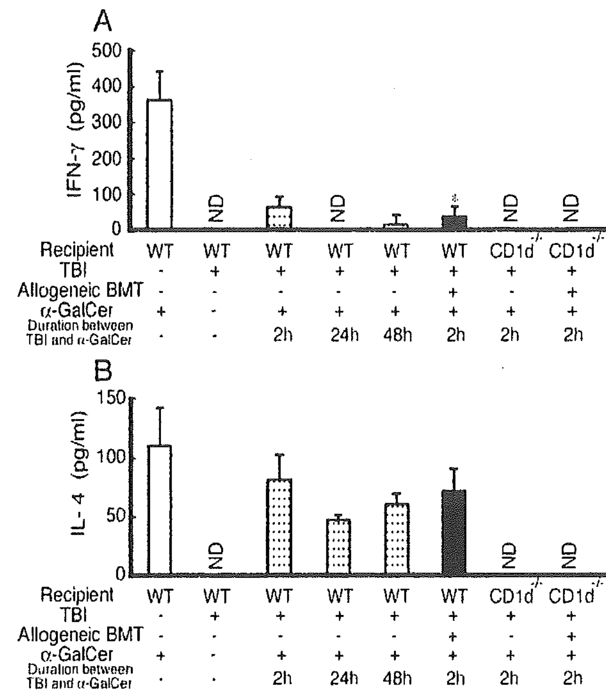

FIGURE 1. Cytokine responses to α-GalCer in lethally irradiated mice with or without BMT. WT and $CD1d^{-/-}$ B6 mice received 13 Gy TBI. Two, 24, or 48 h later, mice were injected i.p. with α-GalCer (100 μg/kg) or diluent. A cohort of animals were transplanted with allogeneic BM cells (5 × 10^6) and spleen cells (5 × 10^6) from WT BALB/c donors immediately after TBI, followed by injection of α-GalCer 2 h after TBI. Six hours after the administration of α-GalCer, serum samples were collected, and levels of IFN-γ (*A*) and IL-4 (*B*) were measured. α-GalCer-treated control mice without TBI (□), recipients of TBI plus α-GalCer (□), and recipients of TBI, allogeneic BMT, and α-GalCer (■) are shown (n = 3 per group). Results represent one of three similar experiments and are shown as mean ± SD. *, $p < 0.05$ vs nonirradiated controls. ND, Not detected.

either syngeneic (B6) or allogeneic (BALB/c) donors. Immediately after BMT, B6 recipients were injected i.p. with either α-GalCer or diluent. Six days after BMT, T cells isolated from mesenteric LN of recipient mice were cultured with irradiated B6 peritoneal cells or with anti-CD3ε mAbs and anti-CD28 mAbs for 48 h, and cytokine levels in the supernatant were determined. Flow cytometric analysis showed that >97% of LN T cells from both control recipients and α-GalCer-treated recipients were donor derived, as assessed by H-2^d vs H-2^b expression. T cells from α-GalCer-treated mice secreted significantly less IFN-γ, but more IL-4, in response to host alloantigens (Fig. 2, *A* and *B*) or to CD3 stimulation (Fig. 2, *C* and *D*) compared with those from controls. Similar results were obtained when T cells isolated from spleens were stimulated by anti-CD3ε and anti-CD28 mAbs. T cells from α-GalCer-treated mice secreted significantly less IFN-γ (18 ± 2 vs 164 ± 6 ng/ml), but more IL-4 (1022 ± 114 vs 356 ± 243 pg/ml), compared with controls. These results demonstrate that a single injection of α-GalCer to BMT recipients polarizes donor T cells toward Th2 responses after allogeneic BMT.

In α-GalCer-treated mice, serum levels of IFN-γ were dramatically reduced on day 6 compared with controls (Fig. 3*A*), and IL-4, which is usually hardly detectable in serum in this model, failed to be detected in the serum of mice of either group (data not shown). This impaired Th1 response of donor T cells was associated with a marked reduction of TNF-α levels in α-GalCer-treated mice (Fig. 3*B*).

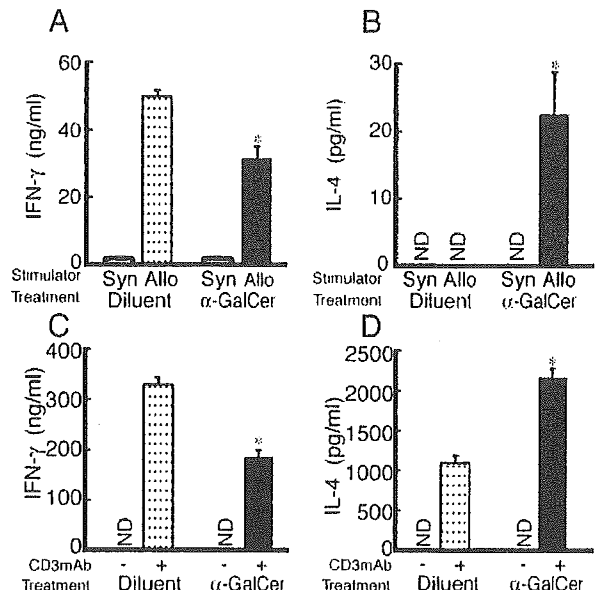

FIGURE 2. Administration of α-GalCer to recipients of allogeneic BMT polarizes donor T cells toward Th2 cytokine secretion. Lethally irradiated (13 Gy) B6 mice were transplanted with BM cells (5 × 10^6) and spleen cells (5 × 10^6) isolated from BALB/c mice, followed by injection of either α-GalCer or control diluent. Mesenteric LN cells obtained from diluent-treated recipients (□) and α-GalCer-treated recipients (■) 6 days after BMT were standardized for numbers of $CD4^+$ T cells as 5 × 10^4/well and were stimulated with 1 × 10^5/well of allogeneic or syngeneic peritoneal cells (*A* and *B*) or with CD3 (*C* and *D*). After 48 h, cytokine levels in the supernatant were measured by ELISA. Results shown are mean ± SD. *, $p < 0.05$ vs diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

Administration of α-GalCer or OCH to BMT recipients modulates acute GVHD

We next examined whether immune deviation mediated by administration of glycolipids can modulate acute GVHD. BMT was performed as above and α-GalCer was injected immediately after BMT on day 0. GVHD was severe in allogeneic controls, with 27% survival at day 50. A single injection of α-GalCer significantly improved survival to 86% ($p < 0.05$) (Fig. 4*A*). Allogeneic control mice developed significantly more severe clinical GVHD compared with syngeneic controls, as assessed by clinical GVHD scores (Fig. 4*B*). Clinical GVHD scores were significantly reduced in α-GalCer-treated recipients compared with allogeneic controls, but were greater than in syngeneic controls. Histological analysis showed that administration of α-GalCer significantly suppressed GVHD pathological scores in the intestine ($p < 0.05$). Analysis of donor cell engraftment at day 60 after BMT in spleens showed complete donor engraftment in α-GalCer-treated recipients (>99% H-$2K^{d+}$/H-$2K^{b-}$ donor chimerism), ruling out rejection or mixed chimerism as a potential cause of GVHD suppression.

Similar protective effects against GVHD were observed in mice treated with OCH, further confirming the protective effects of NKT ligands (Fig. 4*C*). We performed BMT from B6 donors to BALB/c recipients to rule out strain artifacts. Again, a single injection of α-GalCer to BALB/c recipients reduced GVHD and significantly improved survival of animals (Fig. 4*D*).

Host NKT cells and host production of IL-4 are required for suppression of GVHD by α-GalCer

We examined the requirement of host NKT cells in this protective effect of α-GalCer, using NKT cell-deficient $CD1d^{-/-}$ mice as

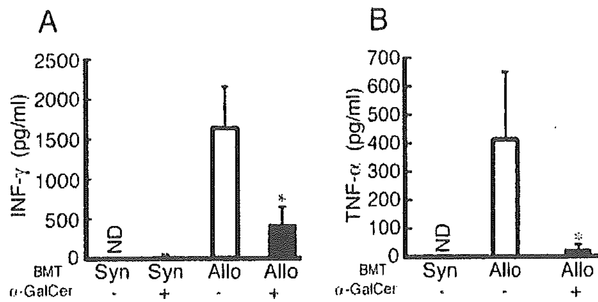

FIGURE 3. A single injection of α-GalCer to recipients of allogeneic BMT markedly reduces serum levels of IFN-γ and TNF-α. WT B6 mice were transplanted as in Fig. 2. Sera (n = 3–10/group) were obtained from diluent-treated (□) and α-GalCer-treated (■) recipients on day 6 after BMT, and serum levels of IFN-γ (*A*) and TNF-α (*B*) were determined. Results from three similar experiments are combined and shown as mean ± SD. *, $p < 0.05$ vs allogeneic, diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

BMT recipients. Lethally irradiated CD1d$^{-/-}$ mice were transplanted with BM cells and spleen cells from WT BALB/c donors, followed by administration of α-GalCer immediately after BMT on day 0. Protective effects of α-GalCer administration were not observed when CD1d$^{-/-}$ B6 mice were used as recipients, confirming the requirement for host NKT cells (Fig. 5*A*). We next examined the requirement of IL-4 production by host cells in this

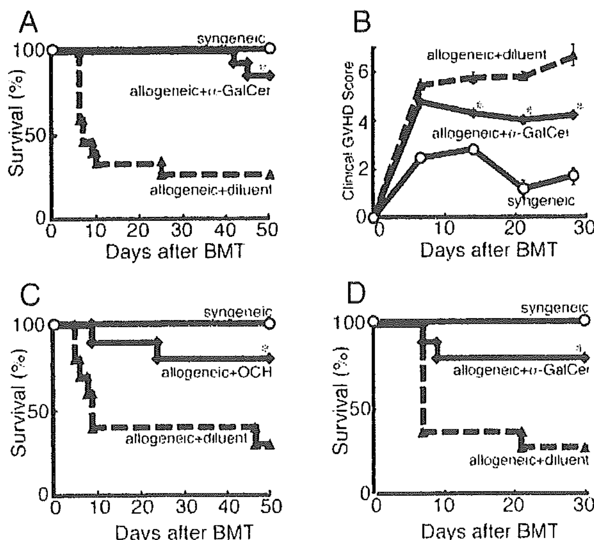

FIGURE 4. A single injection of NKT ligands to BMT recipients modulates acute GVHD. BMT was performed as in Fig. 2. *A*, Survival curves of syngeneic control group (○, solid line; n = 9); allogeneic, diluent-treated recipients (▲, dotted line; n = 15); and allogeneic, α-GalCer-treated recipients (♦, solid line; n = 14) are shown. Data from three similar experiments were combined. *B*, Clinical scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (♦, solid line) are shown as the mean ± SE. *C*, Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, OCH-treated recipients (♦, solid line; n = 10) are shown. Data from two similar experiments were combined. *D*, Lethally irradiated (9 Gy) BALB/c mice were transplanted from B6 donors. Survival curves of the syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (♦, solid line; n = 10) are shown. Data from two similar experiments were combined. *, $p < 0.05$ vs diluent-treated group.

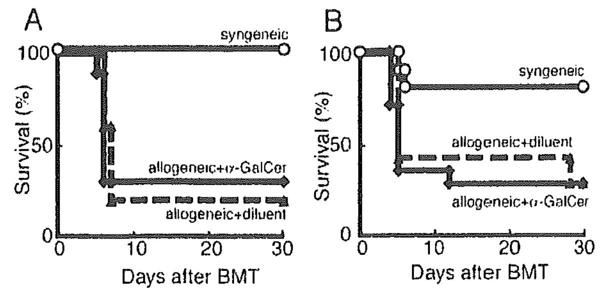

FIGURE 5. Host NKT cells and host IL-4 production are required for suppression of GVHD by α-GalCer. *A*, Lethally irradiated CD1d$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (♦, solid line; n = 10) are shown. Data from two similar experiments were combined. *B*, Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 11); allogeneic, diluent-treated recipients (▲, dotted line; n = 14); and allogeneic, α-GalCer-treated recipients (♦, solid line; n = 14) are shown. Data from three similar experiments were combined.

protective effect. Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted from WT BALB/c donors and administered α-GalCer as above. α-GalCer did not confer protection against GVHD in IL-4$^{-/-}$ recipients (Fig. 5*B*). Taken together, these results indicate that protective effects of α-GalCer are dependent upon host NKT cells and host production of IL-4.

STAT6 signaling in donor T cells is required for modulation of GVHD by α-GalCer

To determine whether IL-4-induced signaling in donor T cells is critical for the protective effect of glycolipids on GVHD, we used donor spleen cells that lack STAT6 and have impaired IL-4 responses (34, 35). Spleen cells from STAT6$^{-/-}$ BALB/c mice and TCD BM cells from WT BALB/c mice were transplanted after lethal TBI, followed by a single injection of α-GalCer. α-GalCer treatment failed to reduce morbidity and mortality of acute GVHD when STAT6$^{-/-}$ BALB/c donors were used (Fig. 6), demonstrating that STAT6 signaling in donor cells is critical for the protective effect of α-GalCer against GVHD.

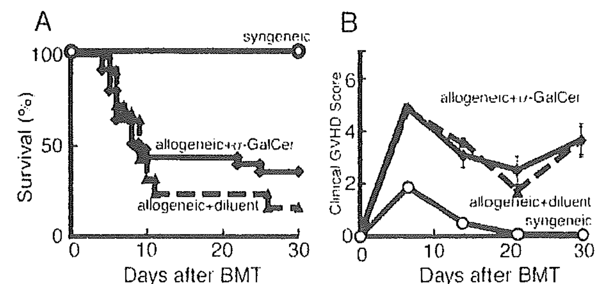

FIGURE 6. The protective effects of α-GalCer against GVHD are dependent upon the STAT6 pathway of donor T cells. Lethally irradiated B6 mice were transplanted with TCD-BM cells (4 × 10^{6}) from WT BALB/c mice and spleen cells (5 × 10^{6}) from STAT6$^{-/-}$ BALB/c mice. *A*, Survival curves of the syngeneic control group (○, solid line; n = 15); allogeneic, diluent-treated recipients (▲, dotted line; n = 25); and allogeneic, α-GalCer-treated recipients (♦, solid line; n = 25) are shown. Data from five similar experiments were combined. *B*, Clinical GVHD scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (♦, solid line) are shown as the mean ± SE.