

Exacerbation of CIDP by IFN β in MS

Table 1. Peripheral Nerve Conduction Study of the Patient before and after Interferon β -1b Therapy

	Motor nerve															Sensory nerve					
	MCV (m/s)			DL (ms)			CMAP (mV)			F-No (%)			F-Lat (ms)			SCV (m/s)			SNAP (μ V)		
	Feb	May	June	Feb	May	June	Feb	May	June	Feb	May	June	Feb	May	June	Feb	May	June	Feb	May	June
R. Median	38 (45~65)	37	36	3.4 (<4.5)	3.8	3.5	13 (4~25)	13	12	NE (>70%)	NE	56	NE (24~29)	NE	44	55 (45~68)	60	61	5 (10~60)	18	23
Ulnar	49 (45~65)	47	45	3 (<4.0)	2.9	2.8	11 (3~25)	8	6	63	NE	NE	32 (24~29)	NE	NE	48 (45~65)	50	53	8 (10~60)	7	8
Tibial	30 (40~60)	46	32	5.1 (<7.5)	6	4.8	34	20	25	100	NE	100	53 (43~52)	NE	68	-	-	-	-	-	-
Peroneal	47 (40~60)	-	-	6.2 (<7.0)	-	-	13 (7~40)	-	-	56	-	-	58 (43~52)	-	-	-	-	-	-	-	-
Sural	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	36	46	40	13 (15~40)	7	12
L. Median	38 (45~65)	34	37	3.6 (<4.5)	3.5	3.3	17 (4~25)	18	17	63 (>70%)	NE	88	32 (24~29)	NE	37	52 (45~68)	61	65	8 (10~60)	20	33
Ulnar	45 (45~65)	38	41	3.3 (<4.0)	3.5	3	9 (3~25)	10	9	44	100	75	34 (24~29)	37	35	50 (45~65)	52	53	10 (10~60)	3	8
Tibial	34 (40~60)	36	35	5.3 (<7.5)	4.8	4.1	38	32	27	100	88	100	59 (43~52)	63	61	-	-	-	-	-	-
Peroneal	48 (40~60)	40	-	6.1 (<7.0)	4.9	-	2.1 (7~40)	4.4	-	44	NE	-	60 (43~52)	NE	-	-	-	-	-	-	-
Sural	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35 (40~60)	42	44	13 (15~40)	12	9

MCV: motor conduction velocity, DL: distal latency, CMAP: compound muscle action potential, F-No: F wave evoked frequency, F-Lat: F wave latency, SCV: sensory conduction velocity, SNAP: sensory nerve action potential, NE: not evoked, -: not examined. Normal values are indicated in parentheses.

with mild weakness, which was resolved within 2 months. In May 1999 she developed right facial palsy followed by mild weakness of the right upper and lower limbs. At that time, tendon reflexes in all four limbs were normal. A brain MRI revealed multiple areas of increased signal intensity on T2-weighted images in the periventricular white matter of the bilateral cerebral hemispheres and some showed gadolinium-enhancement. She was thus diagnosed as having MS and she took oral prednisolone (1 mg/kg) with gradual tapering, after which she almost completely recovered except for minimal numbness of her right hand. In March 2002 she once again developed right facial palsy, and again responded well to oral prednisolone, which brought about complete remittance of her symptoms after three weeks of treatment. Eight months later, she developed left followed by right leg numbness. Neurological examination showed mild weakness in the left lower limb with a mild decrease in superficial sensations in both legs. Tendon reflexes were normal in the upper limbs but mildly hyperactive in the lower limbs. The Babinski sign was elicited in her left lower limb. She was once again treated with oral prednisolone, with complete recovery from leg weakness. However, minimal numbness in the distal parts of her left lower limb persisted. Although her illness clinically responded well to prednisolone, a follow-up MRI obtained one month after initiation of corticosteroids

showed new multiple gadolinium-enhancing white matter lesions in the bilateral cerebral hemispheres. From February 2003, she was therefore started on 8x10⁶ units of IFN β -1b given subcutaneously every other day. Two months later, she showed a progressive worsening of the numbness in her right upper and lower limbs (Table 1 and Fig. 1).

Neurological examinations revealed temporal pallor of the left optic disc and mild weakness and superficial sensory disturbance in the distal parts of the right upper and lower limbs. Right triceps reflex and both sides ankle jerks were absent, and others were hypoactive. The Babinski sign was elicited bilaterally. Laboratory examinations disclosed an increase of liver enzymes [aspartate transaminase: 46 U/l (normal <33 U/l) and alanine transaminase: 101 U/l (normal <30 U/l)] indicating IFN β -1b-induced liver damage. In February 2003 her baseline thyroid function had been normal [TSH: 2.35 μ IU/ml (0.27<normal<4.20) and free-T4: 1.04 ng/dl (1.00<normal<1.80)] with an upper normal limit of anti-thyroid autoantibody titers. However, at this time, after IFN β -1b therapy, she had subclinical compensated hyperthyroidism (TSH: 0.03 μ IU/ml, free-T4 1.43 ng/dl) and elevated serum autoantibodies against human thyroglobulin (TG) and thyroid peroxidase (TPO) [2,094.5 IU/ml (45 in February 2003) and 167.6 IU/ml (7 in February 2003)]. Anti-ganglioside antibodies and other common autoantibodies

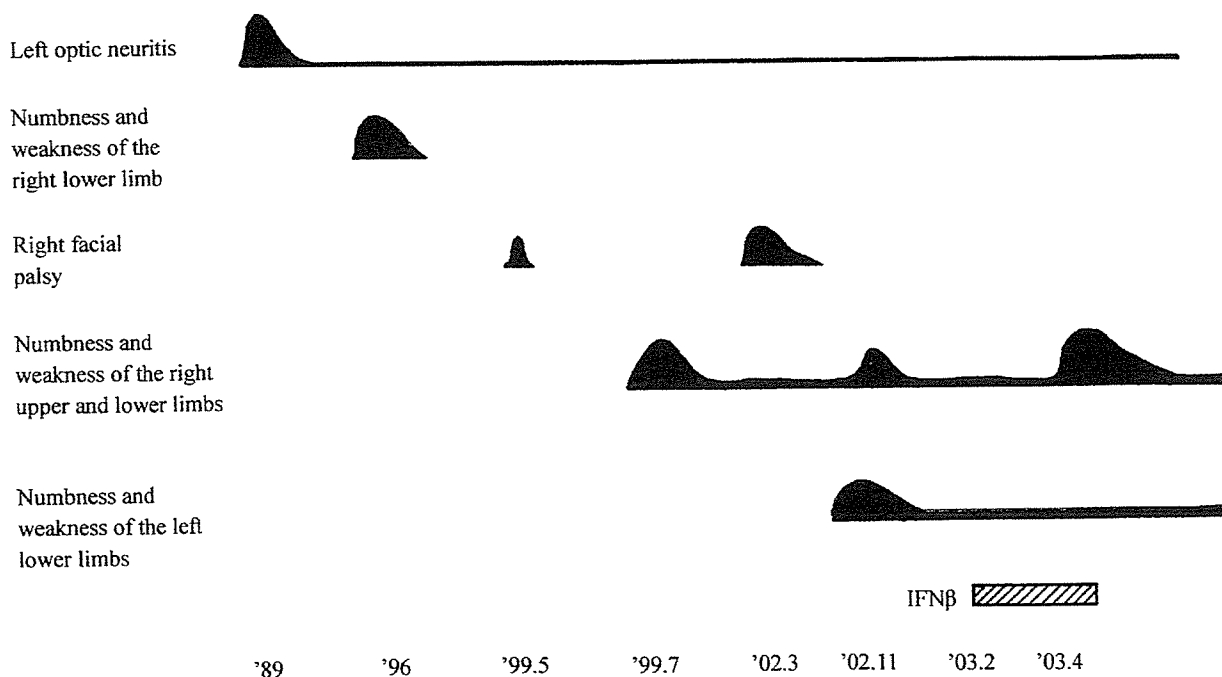


Figure 1. Clinical course of the presented patient.

such as anti-nuclear antibodies, anti-double strand DNA antibodies, anti-RNP antibody, anti-SSA antibody, anti-SSB antibody, anti-Sm antibody and perinuclear and cytoplasmic anti-neutrophil cytoplasmic antibodies were all negative. CSF examination showed 5 nucleated cells/ μ l (all lymphocytes) and an elevated protein level of 81 mg/dl. A baseline conduction study conducted in February 2003 revealed decreased motor conduction velocities and prolonged F wave latencies in several nerves (Table 2), indicating the existence of subclinical demyelinating neuropathy, yet the criteria for CIDP (11) were still not fulfilled. After two months of treatment, however, most of the F waves were not evoked and in the left ulnar and tibial nerves the F wave latencies were markedly prolonged (Table 2). These findings indicated that the demyelinating process had worsened at the nerve roots after IFN β -1b therapy; at this point the patient fulfilled the criteria for probable CIDP (11). As for MS, a brain MRI disclosed multiple small periventricular white matter lesions without gadolinium enhancement (Fig. 2). The number of lesions on the T2-weighted images did not increase compared with those seen in January 2003. A spinal MRI also revealed high signal intensity lesions at the C7 level on the right side and at the T6-7 levels on T2-weighted images (Fig. 2), which were unchanged since February 2003 and not enhanced by gadolinium. Spinal roots including cauda equina were not enhanced by gadolinium. We then discontinued IFN β -1b therapy and treated the patient with oral prednisolone at a dose of 1 mg/kg with a gradual taper. Her weakness was resolved, tendon reflexes were normalized, and sensory

impairment was recovered to her baseline level (Table 1). After corticosteroid therapy, both the F wave evoked frequencies and F wave latencies were markedly improved (Table 2).

Discussion

Although baseline peripheral nerve conduction abnormalities might have partly contributed to the patient's neurologic symptoms prior to IFN β therapy, the preserved tendon reflexes and presence of pathological reflexes together with spinal cord lesions on the MRI suggested that central demyelination was responsible for the earlier neurological symptoms. After IFN β therapy, however, the decreased tendon reflexes and further worsening of peripheral nerve conduction abnormalities suggested that these worsening neurologic symptoms after IFN β therapy were attributable to peripheral demyelination.

The present case is similar to those of Pirko et al (4) in that MS onset occurred in childhood, and that IFN β did not prevent the development of clinically overt CIDP. In their cases, the time lags between the initiation of IFN β and CIDP development were 4 months, 1 year and 4 years, while our patient's CIDP worsened after just 2 months of initiating IFN β therapy, suggesting that in our case IFN β played a role as an exacerbating factor for CIDP. In the present patient, autoimmune thyroiditis also developed after IFN β therapy. IFN β thus appeared to trigger organ-specific autoimmune diseases in our patient. Exacerbation of autoimmune

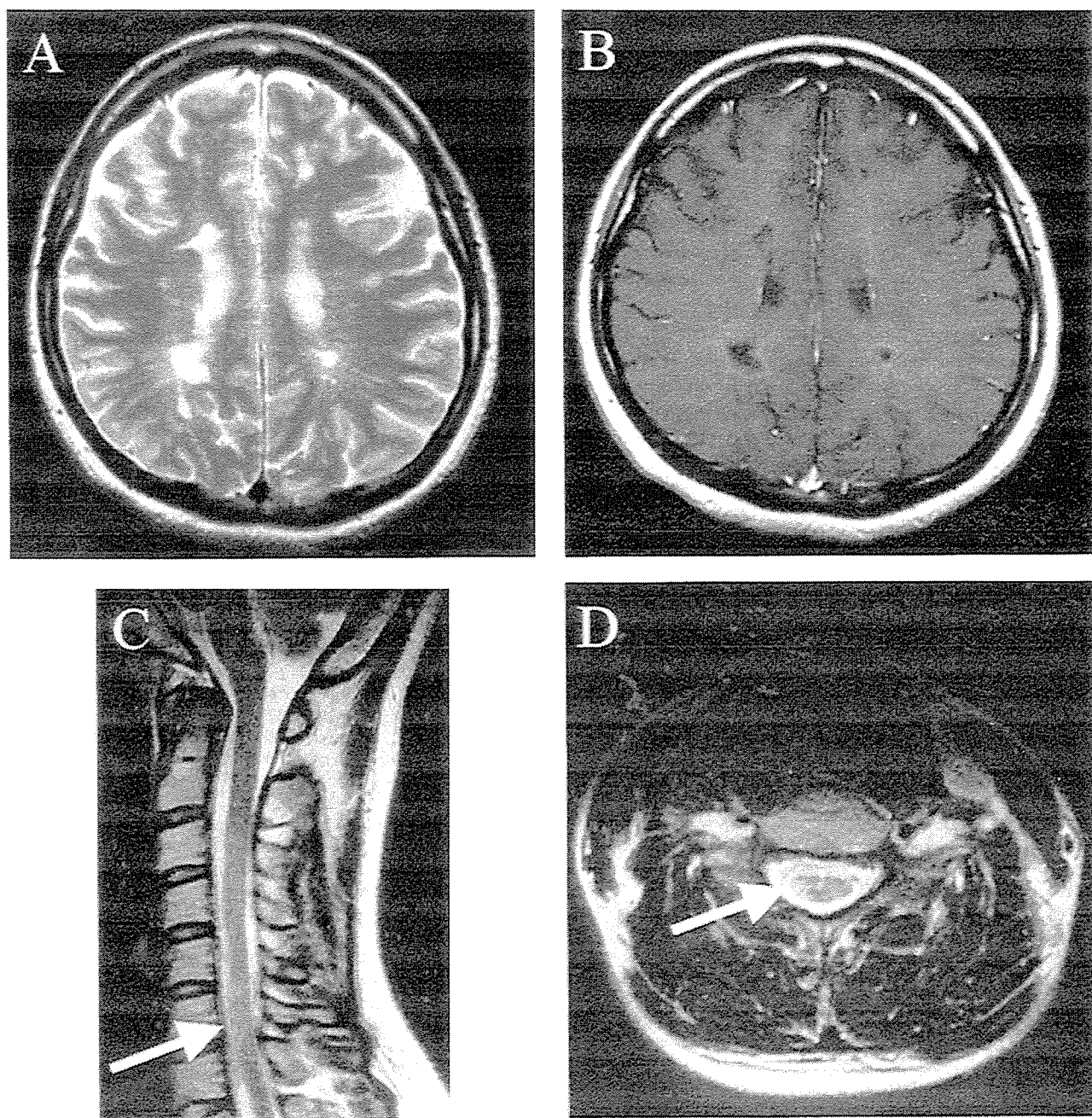


Figure 2. T2-weighted (A) and gadolinium-enhanced T1-weighted (B) brain MRI and T2-weighted spinal cord MRI (C and D) of the patient at the time of CIDP exacerbation. Note the multiple periventricular lesions without gadolinium enhancement. Arrows show the high signal intensity lesions at the C7 level and at the T6-7 levels. The spinal cord MRI also shows a discrete lesion at the C7 spine level that is not enhanced by gadolinium (not shown).

phenomena has been frequently reported with IFN α (12–15) but not for IFN β (16), but both share a common receptor. Dayal et al (17) reported that IFN- γ secreting cells increased in the early course of IFN β therapy. Moreover, it has been demonstrated by micro-array analysis that IFN β therapy upregulates many Th1 genes (18). In CIDP, CXCL-10 (IP-

10), a chemokine that attracts mainly Th1 cells, has been demonstrated to be elevated in CIDP CSF (19, 20). Moreover, T cells bearing CXCR3, which is a chemokine receptor for CXCL10 and specific for Th1 cells, are shown to mainly infiltrate into the biopsied sural nerves (19). Therefore, in CIDP, Th1 cells are thought to play a crucial

role. Therefore, IFN β might also induce autoimmune diseases targeting peripheral nerve myelin through activation of type I cytokines.

The present case together with Pirko's cases (4) suggests that patients with childhood onset MS carry a higher risk of developing CIDP from IFN β administration. Childhood MS reportedly has some distinct clinical and immunological features compared with adult-onset MS, such as a lower frequency of oligoclonal IgG bands in CSF (21), higher CSF cell counts (22), a higher frequency of EEG abnormalities (21), and a higher frequency of relapsing-remitting type (21, 23). Moreover, fever, asthenia, and anorexia are frequently present during the first episode in childhood MS in association with symptoms related to involvement of the spinal cord or cerebellum, suggesting an acute postinfectious autoimmune disorder (22). Such immunological characteristics might also be related to the predisposition for CIDP after IFN β therapy in childhood-onset MS.

The present case further supports the notion that central and peripheral demyelination might have a distinct pathogenic mechanism (4). Although the presence of subclinical peripheral conduction abnormality was uncertain in Pirko's cases (4), initiation of IFN β should be cautiously undertaken in the presence of subclinical demyelinating neuropathy in MS patients, especially in those with childhood onset.

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Autoimmunity against neurofilament protein and its possible association with HLA-DRB1*1502 allele in glaucoma

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Received 11 February 2005; accepted 25 March 2005

Available online 28 April 2005

Abstract

Glaucoma is understood as a neurodegenerative disease and intraocular pressure has been regarded as the major risk factors for the optic nerve damages. However, recent studies suggested that several risk factors including autoimmunity are also shown to play important roles in glaucoma. To identify the retinal antigen in glaucoma, we used the serological analysis of recombinant cDNA expression libraries (SEREX) approach and quantified IgG antibodies directed against the identified antigens in an ELISA. We identified neurofilament protein and the prevalence of anti-bovine neurofilament light subunit (NF-L) autoantibodies in glaucomatous patients was significantly higher than in healthy controls and patients with other uveitic and optic nerve diseases ($P < 0.05$). In addition, our immunogenetic analysis showed a possible association between HLA-DRB1*1502 allele and the patients positive for anti-NF-L autoantibodies. It suggests that the HLA class II-linked gene may be involved in development of autoimmunity in patients with glaucoma.

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Keywords: Glaucoma; Serological analysis of recombinant cDNA expression libraries (SEREX); Neurofilament protein; Autoantibodies; HLA-DRB1*1502 allele

1. Introduction

Glaucoma is classified as a neurodegenerative disease, in which the onset and progression of optic nerve damages results in visual field defects. Intraocular pressure (IOP) has been regarded as the major risk factor for the progression of optic nerve damage. However, recent clinical and experimental studies suggest that several risk factors such as ischemia, glutamate and genetic background also play important roles in the onset and progression of glaucoma [1,2]. These risk

factors are likely associated with the occurrence of apoptotic cell death in retinal ganglion cells in glaucomatous eyes [3,4].

Recently, several investigators have suggested that autoimmunity directed against retinal proteins may be related to the development of glaucomatous optic neuropathy in glaucomatous patients. For example, Wax and his colleagues revealed that various autoantibodies reactive to retinal antigens such as rhodopsin, 27 kDa heat shock protein (hsp27), α - β crystalline, glycosaminoglycans, glutathione *S*-transferase (GST) were found in the sera of glaucomatous patients [5–8]. Also, a recent study using sera obtained from Japanese glaucomatous patients demonstrated the presence of anti- γ -enolase autoantibodies. Interestingly, vitreous

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injection of the anti- γ -enolase autoantibodies resulted in retinal damage in rats [9]. On the other hand, Schwartz and her colleagues reported that T cell-mediated autoimmunity plays a crucial role in the progression of glaucomatous optic neuropathy in mice and rats, and suggested possibility of immunotherapy for neuroprotection against glaucomatous optic neuropathy [10,11]. Taken together, immunological investigation of the glaucomatous optic neuropathy may lead to the development of a novel concept for the treatment of glaucoma in addition to the presently used IOP-lowering treatments.

Herein, we present evidence that neurofilament protein may be a novel autoantigen that is involved in autoimmunity and leads to the development of glaucomatous optic neuropathy. Also, our immunological analysis revealed that there was a statistical association between the HLA class II allele and the presence of autoantibodies directed against neurofilament protein in patients which suggests that there is HLA-linked genetic control of autoimmune glaucoma.

2. Materials and methods

2.1. Patients

In this study, 65 patients with glaucoma were subjected to analysis. The mean age (\pm S.D.) in the patients was 61.3 ± 12.6 years old (range, 22–78 years old). The patients included 34 males and 31 females. In 30 of the 65 patients, the glaucoma was diagnosed as normal tension glaucoma (NTG) based upon (1) open iridocorneal angles without any peripheral anterior synechia, (2) no evidence of elevation in intraocular pressure (>21 mmHg), (3) the presence of glaucomatous (or glaucoma-like) changes in visual fields and optic nerve appearance and (4) exclusion of any other causes of optic neuropathy. The remaining 35 patients were diagnosed as having primary open angle glaucoma with glaucomatous optic neuropathy and had a history of high intraocular pressure (>21 mmHg). Additionally, in this study, 42 patients with Vogt–Koyanagi–Harada disease (VKH), 31 patients with Behçet's disease (BD) with ocular complications, 30 patients with the opticospinal form of multiple sclerosis (OS-MS) and 30 patients with the conventional form of multiple sclerosis (C-MS) were subjected to immunological analysis. Sixty-nine normal healthy volunteers were used as healthy controls in the present study. Peripheral venous blood samples were immediately subjected to serum separation and stored at -80°C before use. All subjects were unrelated Japanese living in Japan and informed consents were obtained from all subjects prior to blood sampling. This study was done according to the tenets of the Declaration of Helsinki.

2.2. Immunoscreening

A rat retinal cDNA expression library was purchased from Stratagene (La Jolla, CA, USA). The immunoscreening

method used was a modification of our published methods [12,13]. Sera were diluted in Tris-buffered-saline (TBS). Recombinant phages at a concentration of $1 \times 10^4/15$ cm plate were amplified for 6 h at 42°C , then transferred onto nitrocellulose filters Hybond-c extra (Amersham, Buckinghamshire, England), pretreated with isopropyl b-D-thiogalactoside (Wako, Osaka, Japan), and incubated for an additional 3 h at 37°C to transfer the encoded proteins onto the filter membranes. Membranes were then removed from plate and blocked with 5% (w/v) skim milk/TBS. After washing with TBS containing 0.05% Tween 20 (TBS-T), membranes were incubated in 2 h at 27°C . This was followed by incubation in horseradish peroxidase (HRP)-conjugated mouse anti-human IgG (Southern Biotechnology Associates Inc., Birmingham, AL) for 1 h at room temperature. The membranes were washed in TBS-T and TBS and incubated with ECL RPN 2106 (Amersham) for 1 min, and exposed to autoradiographic film to detect any antibody-reactive phage plaques. Positive recombinant clones were subcloned and retested for sera reactivity as described above. A total of 1.5×10^6 recombinant clones were screened, using sera from three glaucomatous patients.

2.3. Sequence analysis of identified cDNA clones

Immunoreactive phage clones were subjected to *in vivo* excision of pBluescript phagemid, using the ExAssist helper phage/SOLR strain system (Stratagene). Plasmid DNA was purified, using QIAGEN Plasmid Midi Kits (Tokyo, Japan) cDNA inserts were sequenced, using an ABI Prism (Perkin-Elmer, Norwalk, CT) automated DNA sequencer and for sequence alignments we used BLAST software on NCBI and Genone Net.

2.4. Enzyme-linked immunosorbent assay

Detection and titration of antibodies reactive to purified bovine neurofilament protein (PROGEN, Heidelberg, Germany) were done, using a direct enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96-well) (NUNC, Denmark) were coated with neurofilament protein in PBS (pH 7.4) for 10 h at 4°C . The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 5% skim milk/PBS for 2 h at room temperature. The plates were washed with PBS-T and incubated for 2 h at room temperature with serum samples diluted at 1:100 with 1% skim milk/PBS. The plates were washed in PBS-T and 100 μl of HRP-conjugated mouse anti-human IgG diluted at 1:2000 with 1% skim milk/PBS was added to each well and then followed by incubation at room temperature for 1 h. The plates were washed with PBS-T, and 100 μl solution of *o*-phenylenediamine (Sigma Fast: Sigma Chemical Co., St. Louis, MO) was added to each well. After 15 min, the reaction was stopped by adding 100 μl of 4M H_2SO_4 , and the optical density (OD) at 490 nm was determined, using a Model 550 microplate reader (Bio-Rad, Hercules, CA). The reactiv-

ities of autoantibodies are expressed as the relative value of the OD unit in which the OD value for the serum sample of patient TT was estimated to be 100 OD units.

2.5. HLA-DRB1 typing

Extraction of genomic DNAs was performed by sodium dodecyl sulphate and proteinase C treated peripheral blood cells from 55 patients with glaucoma and 57 healthy controls. HLA-DRB1 genes were amplified by polymerase chain reaction (PCR) method and typed by the PCR-restriction fragment length polymorphism (RFLP) method as described elsewhere [14].

3. Results

3.1. Identification of immunoreactive cDNA clones

With a serum sample obtained from a patient with NTG (a 58-year-old man), 4.0×10^5 phage plaques in the rat retinal cDNA library were immunoscreened. Seven positive immunoreactive clones were identified. Their partial sequences of inserted DNAs and subsequent computer analysis revealed that they were derived from two different sequences. The deduced amino acid sequences of six clones were identical to rat neurofilament protein and another clone encoded for the Wiskott-Aldrich syndrome proteins family verprolin homologous protein3 (WAVE3). Thus, our immunoscreening studies revealed the presence of anti-rat neurofilament protein IgG antibodies in a patient with NTG.

3.2. Comparison of the prevalence of anti-bovine neurofilament IgG in sera from patients with glaucoma, other diseases and healthy controls

To determine if the autoimmunity directed against the neurofilament protein is associated with glaucomatous optic nerve damage, serum samples obtained from 65 glaucomatous patients, 42 patients with VKH, 31 patients with BD, 60 patients with MS and 69 healthy controls were tested for their reactivity to neurofilament protein. Because purified human neurofilament protein was not available for the present studies, bovine neurofilament protein (68-kD light subunit) was purchased from PROGEN (Heidelberg, Germany) and was used for the following experiments to determine the levels of antibody titers in ELISA in sera from the various patients and normal volunteers. Computer analysis of the deduced amino acid sequences demonstrated by the homology of the 68-kD light subunit of neurofilament (NF-L) between bovine and human, human and rat, rat and bovine was 78–81%.

Fig. 1 shows scatter grams of individual serum titers of the anti-NF-L IgG. The results were expressed as the relative value of OD unit. To determine the cut-off level for positivity of anti-NF-L in the 69 healthy controls, titer levels were measured. The mean (\pm S.D.) titer level of the anti-NF-L IgG

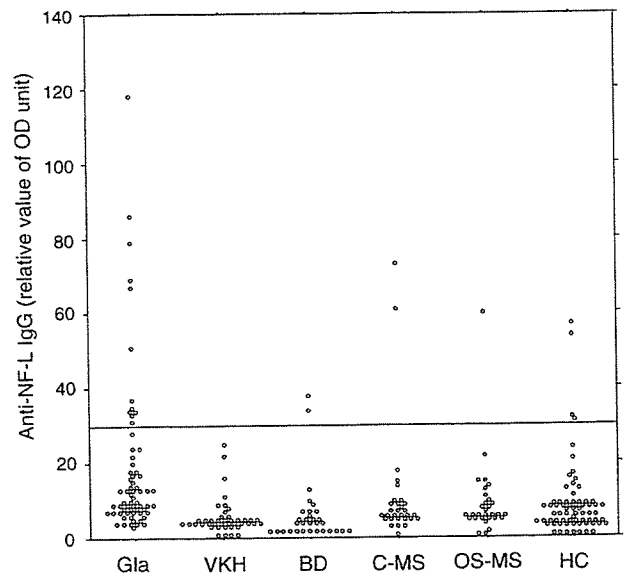


Fig. 1. Distribution of the titer of anti-bovine NF-L IgG antibodies in patients with glaucoma (Gla), Vogt-Koyanagi-Harada disease (VKH), Behçet's disease (BD), opticospinal form of multiple sclerosis (OS-MS), conventional form of multiple sclerosis (C-MS) and healthy controls (HC). Data were expressed as the relative value of optical density (OD) unit in which the OD value for the serum sample of patient TT was estimated to be 100 OD units. To determine the cut-off level for the positivity of anti-NF-L IgG, the levels were measured in 69 healthy controls. The mean (\pm S.D.) titer level of the anti-NF-L IgG was 7.5 ± 8.7 . Thus, we regarded the mean value plus two times the standard deviation (24.9), as the cut-off level for this ELISA analysis (indicated by the line).

was 7.5 ± 8.7 . Thus, we regarded the mean value plus two times the standard deviation, $7.5 + 8.7 \times 2 = 24.9$, as the cut-off level for this ELISA analysis. The prevalence of anti-NF-L IgG in patients and healthy controls is shown in Table 1. Our ELISA experiments showed that the measured titer levels of anti-NF-L IgG were higher than the cut-off level, found in 12 (18.5%) of the 65 glaucomatous patients and in four (5.8%) of the 69 normal controls ($P = 0.022$, Fisher's exact test). The difference in the percentage of autoantibody-positive subjects was statistically significant between these two groups.

Table 1
Prevalence of anti-NF-L IgG evaluated by ELISA in sera from patients with glaucoma, disease controls and healthy controls

Diagnosis	Anti-NF-L IgG positive donors	Frequency (%)
Glaucoma	12/65	18.5*
VKH	0/42	0.0
BD	2/31	6.5
OS-MS	1/30	3.3
C-MS	2/30	6.7
HC	4/69	5.8*

VKH, Vogt-Koyanagi-Harada disease; BD, Behçet's disease; OS-MS, opticospinal form of multiple sclerosis; C-MS, conventional form of multiple sclerosis; HC, healthy control.

* Statistically significant difference in frequency of anti-NF-L IgG autoantibodies between HC and glaucom groups ($P = 0.022$, Fisher's exact test).

Table 2

Allele frequency of HLA-DRB1 in patients with glaucoma and normal controls

Allele	Glaucoma (n=55)		Control subject (n=57)		P-value
	n	AF	n	AF	
DRB1*0101	8	0.145	9	0.158	NS
DRB1*0301	1	0.018	1	0.018	NS
DRB1*0401	1	0.018	1	0.018	NS
DRB1*0403	2	0.036	2	0.035	NS
DRB1*0405	16	0.291	12	0.211	NS
DRB1*0406	4	0.073	5	0.088	NS
DRB1*0410	2	0.036	2	0.035	NS
DRB1*0802	4	0.073	3	0.053	NS
DRB1*0803	10	0.182	12	0.211	NS
DRB1*0901	17	0.301	15	0.263	NS
DRB1*1101	1	0.018	3	0.053	NS
DRB1*1201	1	0.018	4	0.07	NS
DRB1*1301	1	0.018	1	0.018	NS
DRB1*1302	8	0.145	9	0.158	NS
DRB1*1401	2	0.036	0	0	NS
DRB1*1405	4	0.073	3	0.053	NS
DRB1*1406	3	0.055	0	0	NS
DRB1*1501	8	0.145	8	0.14	NS
DRB1*1502	16	0.291	14	0.246	NS
DRB1*1602	1	0.018	1	0.018	NS

AF: allele frequency; NS: not significant; Chi-square test.

We measured antibody titer levels of anti-NF-L IgG in serum samples obtained from patients with various ocular diseases. ELISA studies showed that the titer levels higher than the cut-off value were found in none (0%) of the 42 patients with VKH, two (6.5%) of the 31 patients with BD, one (3.3%) of the 30 patients with OS-MS and two (6.7%) of the 30 patients with C-MS. As shown in Table 1, there were no statistically significant differences in the prevalence of the titer levels of anti-NF-L IgG that were higher than the cut-off level between the normal controls and each group. The age distribution is not significantly different between anti-NF-L IgG positive and negative donors in each group of patients as well as healthy controls (data not shown).

3.3. HLA-DRB1 allele frequency

There were no statistically significant differences in the allele frequency of HLA-DRB1 between normal controls and the combined glaucomatous patient group (Table 2). We divided the glaucomatous patients into two groups based upon their anti NF-L IgG titer levels. In the anti NF-L IgG positive group, which was composed of patients with anti NF-L IgG titer levels higher than the cut-off level, HLA-DRB1*1502 allele was observed in seven (58.3%) of the 12 patients. On the other hand, in the anti-NF-L IgG negative group, which was composed of patients with anti-NF-L IgG titer levels equal to or below the cut-off level, the allele was only found in seven (16.3%) of the 43 patients. The differences in prevalence of anti NF-L IgG were statistically significant between these two groups ($P < 0.05$, Chi-square test) (Table 3).

Table 3

Difference between anti-NF-L IgG positive patients and negative patients in frequency of DRB1 alleles

Allele	Anti-NF-L IgG positive patients (n=12)		Anti-NF-L IgG negative patients (n=43)		P-Value
	n	Frequency	n	Frequency	
DRB1*0101	1	0.083	5	0.116	NS
DRB1*0301	0	0.000	1	0.023	NS
DRB1*0401	0	0.000	1	0.023	NS
DRB1*0403	0	0.000	2	0.047	NS
DRB1*0405	4	0.333	10	0.233	NS
DRB1*0406	0	0.000	4	0.093	NS
DRB1*0410	0	0.000	2	0.047	NS
DRB1*0802	0	0.000	4	0.093	NS
DRB1*0803	2	0.167	6	0.14	NS
DRB1*0901	2	0.167	11	0.256	NS
DRB1*1101	1	0.083	0	0	NS
DRB1*1201	0	0.000	1	0.023	NS
DRB1*1301	1	0.083	0	0	NS
DRB1*1302	2	0.167	6	0.14	NS
DRB1*1401	0	0.000	2	0.047	NS
DRB1*1405	1	0.083	3	0.07	NS
DRB1*1406	2	0.167	1	0.023	NS
DRB1*1501	1	0.083	7	0.163	NS
DRB1*1502	6	0.500	9	0.21	<0.05
DRB1*1602	0	0.000	1	0.023	NS

NS: not significant; Chi-square test.

4. Discussion

Recent studies have revealed that glaucomatous optic neuropathy may be caused by many risk factors such as ischemia, genetic background, glutamate and immune response in addition to intraocular pressure [1]. Among these, involvement of the immune response in the pathogenesis of glaucomatous optic neuropathy has been the focus of investigations. Several investigators have reported the presence of autoantibodies in glaucomatous patients [5–8,15,16]. Also, recent studies have suggested that T cell-mediated immune response might play a crucial role in the progression of glaucomatous optic nerve damages in animal experimental models [10,11,17,18]. These findings have shed light on new aspects for understanding of the pathogenesis and treatment of glaucomatous optic neuropathy. Based on these findings, we conducted a series of experiments in the present study in an attempt to further elucidate a role for involvement of autoimmunity and the immunogenetic background, in glaucomatous optic nerve damage.

In previous studies, Western blot analysis was used for the identification of autoantibodies in serum samples obtained from glaucomatous patients [5–8,16]. These methods are useful for identification of autoantibodies directed against antigens that have a large amount of expression. However, this method has a potential risk in that it can exclude the detection of important antigens if the autoantigens are barely expressed. For this reason it is possible that autoantigens associated with the progression of glaucomatous optic neuropathy

may escape detection of a Western blot analysis. Thus, in order to better understand the immunological mechanisms of glaucomatous optic neuropathy, more sensitive and reliable technique needs to be applied. In the present study, we applied SEREX method to the identification of glaucoma-associated autoantigens. This molecular biological method enabled us to detect autoantigens regardless of whether the expression was very low, and to easily accomplish subsequent molecular cloning of genes that encode for the autoantigen.

With the aid of the SEREX method in our previous studies, we have been able to identify disease-associated autoantigens in patients with VKH and cancers [12,19–21]. In VKH, T helper 1 type T cells-mediated cellular immunity plays a dominant role in the pathogenesis [22,23]. In cases of glaucoma, some investigators have suggested the involvement of T cell-mediated cellular immunity play a role in the neuroprotection against glaucomatous optic neuropathy whereas T helper 2 type T cells-mediated humoral immunity should be regarded as the main cause for the production of autoantibodies [11,17]. Thus, presently, our understanding on immunological mechanisms associated with glaucomatous optic neuropathy is far from satisfactory. Therefore, we considered the use of SEREX to be suitable for our aims of elucidating the mechanism of autoimmunity in glaucoma.

In the present study, we identified neurofilament proteins as the candidate target autoantigens associated with glaucoma. Neurofilament proteins are known to be major intermediate filaments that are present in the nervous system and construct the axonal cytoskeleton. So far, three types of subunits for neurofilament have been identified; the 68-kD light subunit (NF-L), the 160-kD medium subunit (NF-M) and the 200-kD heavy subunit (NF-H). NF-L constructs the backbone of neurofilament while NF-M and NF-H are localized on the periphery of NF-L. Targeted disruption of the NF-L gene results in reduction of radial growth of neuronal axons [24]. It has been reported that abnormal accumulation of neurofilament is the pathological hallmark of some neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Lewy-body-type dementia and Parkinson's disease [25–27]. The presence of autoantibodies directed against NF proteins has been reported in patients with progressive MS [28,29]. From the viewpoint of glaucoma, it has been reported that expression of NF proteins in retinal ganglion cells reduced in glaucomatous patients [30,31].

In our study, use of the SEREX method and a serum sample of a patient with normal tension glaucoma, allowed us to identify neurofilament protein as a candidate autoantigen associated with glaucomatous optic neuropathy. Our results showed a statistically significant difference between the prevalence of anti-NF-L IgG in normal controls and glaucomatous patients, but not between normal controls and other diseases such as VKH, BD and MS. In glaucoma, retinal ganglion cells and their axons specifically deteriorate whereas it is the uveal tissues that are mainly involved in VKH. However, in BD and MS as well as glaucoma, optic nerve damage dose occur. Thus, our positive results that showed

the prevalence of a high titer of anti-NF-L IgG in glaucomatous patients might suggest that this immune response may be associated with a certain sub-group of glaucomatous patients. One of the most important points that need to be addressed concerns what mechanisms segregate this sub-group of glaucomatous patients with regards to the immunological reaction against NF-L. This question may be partially answered by our additional results for the typing of HLA class II alleles in glaucomatous patients.

The association between glaucoma and HLA class II allele has been previously reported [32,33]. In the present study, we document the frequency of HLA-DRB1 allele in Japanese glaucomatous patients for the first time. A statistically significant difference was revealed in the allele frequency of HLA-DRB1*1502 between the anti-NF-L IgG positive and negative groups in glaucomatous patients. Our finding suggests that specific HLA class II alleles or genes in with a strong linkage with disequilibrium with HLA-DRB1*1502 may play an important role in the production of autoantibodies in glaucomatous patients. The presence of autoantibodies reactive to retinal and/or optic nerve antigens may result from prolonged exposure of the immune system to autoantigens caused by glaucomatous optic nerve damage. However, we cannot exclude another possibility that the autoantibody-associated immune response may deteriorate (or inhibit) the progression of glaucomatous optic neuropathy. Taken into consideration together, we can hypothesize that the immunogenetic background associated with HLA class II alleles may control susceptibility to autoimmunity. Our hypothesis may shed new light for understanding the presence of autoantibodies in glaucomatous patients. Further studies are necessary to elucidate the relationships between HLA class II alleles and the immunological abnormality in glaucoma.

In conclusion, we have identified neurofilament proteins as a novel autoantigens associated with glaucomatous optic nerve damage. This autoimmunity was found in some but not all glaucomatous patients, and was not found in patients with other ocular diseases. HLA-DRB1*1502 allele was also significantly associated with high titer of anti-NF-L IgG in glaucomatous patients, suggesting the importance of HLA class II-linked genes in the development of autoimmunity in glaucoma.

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Reconstitution and Function of *Tetragenococcus halophila* Chaperonin 60 Tetradecamer

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Received 14 July 2004/Accepted 5 October 2004

Tetragenococcus halophila originally isolated from soy sauce is a halophilic lactic acid bacterium which can grow under 4 M sodium chloride. *T. halophila* chaperonin composed of a core moiety of chaperonin 60 (cpn60) and a lid moiety of chaperonin 10 (cpn10), is thought to contribute to host halotolerant capability. In this report, we reconstituted and characterized the core complex of *T. halophila* chaperonin by using a recombinant *T. halophila* cpn60 (Tcpn60) over-expressed in *Escherichia coli*. The reconstitution of Tcpn60 was performed in the presence of 10 mM MgCl₂, 2 mM ATP and 0.8 M (NH₄)₂SO₄ and the resultant oligomer was purified by gel filtration chromatography. Electron microscopy of the reconstituted Tcpn60 revealed a double toroidal tetradecameric structure that is characteristic of bacterial cpn60. The *T. halophila* tetradecamer cpn60 exhibited an ATPase activity and a refolding activity of both chemically and thermally denatured enolases under wide range of salt concentrations. Furthermore, we demonstrated that heterologous expression of Tcpn60 allowed the normal growth of host *Escherichia coli* cells under salt stress conditions and this effect was further enhanced by co-expression with Tcpn10. These results suggested that Tcpn60 contributes to the halotolerance property of *T. halophila* cell as a tetradecamer complex, probably associated with the Tcpn10 complex.

[Key words: chaperonin 60, halophilic, *Tetragenococcus halophila*, GroEL, reconstitution]

The lactic acid bacterium *Tetragenococcus halophila* is a tetrad-forming gram-positive coccus showing moderately halotolerance. *T. halophila* can grow under salt concentrations ranging from 0.5% (0.086 M) to 30% (5.1 M) (10% [1.71 M] is optimum) and is suitable for use in brine fermentations such as the brewing of soy sauce (1). However, little is known about the halotolerance mechanism of moderately halophilic bacteria, particularly lactic acid bacteria. Robert *et al.* (2) have indicated that compatible solutes such as glycine betaine prevent the accumulation of sodium ions to a level inhibiting growth. They also showed that the concentration of potassium ions instead of sodium ions increased under high salinity conditions with compatible solutes. This suggested that the intracellular environment still suffers from high salinity even with protection by compatible solutes. We are interested in the mechanism of how cellular proteins can be protected from denaturing by high salinity in *T. halophila* cells and have studied on the machinery of *T. halophila* molecular chaperones (3–5).

Chaperonins are molecular chaperones and are a family of ubiquitous proteins highly conserved among almost all organisms from prokaryotes to eukaryotes (6). The function

of chaperonins is to assist correct folding of nascent proteins or refolding of denatured proteins (7, 8). In addition to this function, chaperonins contribute to the maintenance of cell homeostasis, under various conditions including high salinity.

The structure and function of chaperonins have been well studied in *Escherichia coli*, GroEL that comprises identical subunits of tetradecamer GroEL (referred to as Tcpn60 in *T. halophila*, formerly GroEL in Ref. 3) and heptamer GroES (Tcpn10 in *T. halophila*, formerly GroES in Ref. 3). The tetradecamer GroEL consists of two heptameric rings stacked back to back on top of each other (9, 10). The GroEL complex forms a cylinder and makes an interior dome cavity with a lid-like component of the GroES complex, which affords an important space for the chaperonin function (11).

In order to study the function of chaperonins, it is indispensable to obtain the correct form of the chaperonin oligomeric complex, which is the tetradecamer core moiety of the cpn60 cylinder as a minimum unit with chaperonin activity. In the present study, we realized the reassembly of recombinant *T. halophila* cpn60 (Tcpn60) to the tetradecamer cylindrical complex showing ATPase and refolding activities under a wide range of salt concentrations. The functionality of Tcpn60 was also examined together with that of Tcpn10 *in vivo* by heterologous expression in *E. coli*.

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MATERIALS AND METHODS

Plasmids and strains *E. coli* strains used in this study are listed in Table 1. To construct plasmid pET-CPN60, the Tcnp60 gene was amplified from genomic DNA isolated from *T. halophila* JCM5888 by PCR with a set of primers, Tcnp60-F (5'-TCAGAA GCATATGGCAAAAAGATATTAATTTTCAG-3') and Tcnp60-R (5'-TCGCTCGCTCGAGTACTGTTCA-3'). For pET-CPNS, a fragment corresponding to the Tcnp10-Tcnp60 operon was amplified with the primers, Tcnp10-f (5'-TTGGAGGGACATATGGTGTAAACCATTA-3') and Tcnp60-R. Each PCR product was digested by *Nde*I and *Xho*I and ligated into the pET-14b expression vector (Novagen, Madison, WI, USA) treated with the same restriction enzymes. The resultant plasmids were transformed into *E. coli* BL21(DE3) (Novagen).

Expression of Tcnp60 gene in *E. coli* *E. coli* TH-CPN60 was grown at 37°C in 2 × YT medium (1.6% tryptone, 0.5% yeast extract and 0.5% NaCl) supplemented with 50 µg/ml ampicillin. When the absorbance at 600 nm of the culture reached 0.5–0.6, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to final concentration of 1 mM. After additional incubation for 3 h at 37°C, the cell pellets were harvested by centrifugation at 9000 × g for 20 min and stored at –80°C.

Purification of recombinant Tcnp60 The cell pellets were resuspended in 2 volumes of 20 mM Tris–HCl (pH 7.4) containing 10 mM imidazole, 500 mM NaCl, 1 mM PMSF and 10 µg/ml leupeptin. After sonication, cell debris was removed by centrifugation at 9000 × g, the supernatant was loaded onto a nickel affinity column (Amersham Pharmacia Biotech, Uppsala, Sweden) and the column was subsequently washed with 10 ml each of the following buffers: 20 mM Tris–HCl (pH 7.4), 10 mM imidazole, 500 mM NaCl; 20 mM Tris–HCl (pH 7.4), 10 mM imidazole, 200 mM KCl, 2 mM ATP, 5 mM MgCl₂; 20 mM Tris–HCl (pH 7.4), 30 mM imidazole, 500 mM NaCl. In the washing step, MgATP was added to remove any bacterial DnaK bound to the recombinant Tcnp60 (12). The proteins bound to the column were eluted with 20 mM Tris–HCl (pH 7.4) containing 500 mM NaCl and 500 mM imidazole. Fractions containing His₆-tagged Tcnp60 were pooled and applied to a Sephadex G-50 (Amersham Pharmacia Biotech) column (1 × 15 cm) or dialyzed with Slide A-lyzer cassette (Pierce, Rockford, IL, USA) in 50 mM Tris–HCl (pH 7.4), 10 mM MgCl₂ and 100 mM NaCl (buffer A) and then concentrated by ultrafiltration (Millipore, Bedford, MA, USA). The concentrated sample of His₆-tagged Tcnp60 was then applied to a TSK G4000SW_{XL} gel filtration column (7.8 mm, inner diameter × 30 cm; Tosoh, Tokyo) equilibrated with buffer A. The column was developed by the same buffer at flow rate of 0.5 ml/min and monitored by ultraviolet absorption at 280 nm. The maximum volume injected was 100 µl. Fractions (0.5 ml each) containing monomeric Tcnp60 were collected and concentrated to >32 µM by ultrafiltration (Millipore) for subsequent use in the reconstitution experiment.

Reconstitution of Tcnp60 Reconstitution experiments of the Tcnp60 monomer into the oligomer were performed according to the methods previously reported (13–15) with some modifications. Briefly, the concentrated monomeric Tcnp60 (>32 µM protomer) was mixed with a solution containing Tris–HCl (pH 7.4), MgCl₂ and ATP so that the final concentrations were 50 mM, 10 mM and 2 mM, respectively. Then, ammonium sulfate was added gradually

to the monomeric cpn60 solution to a final concentration of 800 mM and the mixture incubated for 60 min to promote reassembly of Tcnp60. Additional ammonium sulfate was then added to be 60% saturation to precipitate the oligomer. After centrifugation, the Tcnp60 oligomer pellet was dissolved in 50 mM Tris–HCl (pH 7.4), 10 mM MgCl₂, 100 mM NaCl and 0.2 mM ATP (buffer B). Then, the reconstituted oligomers were isolated by TSK G4000SW_{XL} gel filtration column chromatography at a flow rate of 0.5 ml/min with buffer B.

Determination of native molecular weight The native molecular weight of the Tcnp60 was determined by TSK G4000SW_{XL} gel filtration column chromatography at a flow rate of 0.5 ml/min with a molecular weight standards kit (Sigma, St. Louis, MO, USA) including thyroglobulin (669,000), apoferritin (440,000), alcohol dehydrogenase (150,000) and carbonic anhydrase (29,000).

Electron microscopy The morphology of the reconstituted Tcnp60 was examined by negative-staining using 0.5% uranyl acetate in a JEM 2000EX electron microscope (JEOL, Tokyo) at 100 kV.

Secondary structure of the recombinant Tcnp60 The purified Tcnp60 oligomer (4.1 µM protomer) and monomer (3.6 µM protomer) were dialyzed against 50 mM Tris–HCl (pH 7.5) and analyzed with a JASCO J-720 spectropolarimeter (JASCO, Tokyo) using a cuvette with a path length of 0.1 cm (far-UV) at 25°C. Ellipticity was measured from 200 to 250 nm at 1-nm intervals, corrected for buffer base lines and converted to molar ellipticity (θ) that is expressed as deg · cm² · dmol⁻¹.

ATPase activity The ATPase activity of Tcnp60 was measured by quantitation of Pi released from ATP using a modified malachite green assay (16). The assay mixture contained 50 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 2 mM ATP, 50 mM KCl and 36 µg of the purified Tcnp60 oligomer and monomer. The reactions were terminated by the addition of perchloric acid after incubations at 30°C for 0, 10, 20 and 30 min. The samples were put on ice and centrifuged briefly. The inorganic phosphate produced by ATP hydrolysis was quantitated by mixing the sample with malachite green and ammonium molybdate solution followed by measuring the absorbance at 620 nm using 96-well microplates. All data presented were corrected for spontaneous ATP hydrolysis under the conditions tested.

Salt requirement for Tcnp60 ATPase activity To determine the salt-dependence of Tcnp60 ATPase activity, Tcnp60 was added to 50 mM Tris–HCl, pH 7.5, containing 10 mM MgCl₂ and 50 mM KCl at 30°C and supplemented with various salts as indicated in the legend of Fig. 5. Activity at 10 mM MgCl₂ and 50 mM KCl is set to 100%.

Refolding assay of guanidine–HCl denatured enolase Yeast enolase (26 µM; Sigma) was denatured with 8 M guanidine–HCl (Gdn–HCl) and 2 mM DTT in 50 mM Tris–HCl (pH 7.5) at 30°C for 30 min. The denatured enolase was rapidly diluted with 50 mM Tris–HCl (pH 7.5) containing 10 mM Mg acetate, 20 mM KCl and 2 mM DTT so that the final concentrations of enolase and Gdn–HCl were 0.2 µM and 60 mM, respectively, in the absence and presence of the indicated concentration of NaCl. Refolding of the denatured enzyme was done at 30°C with the reconstituted Tcnp60 tetradecamer and ATP at the respective final concentrations of 0.4 µM and 2 mM. The final volume of the refolding mixture was 500 µl. The activity of the refolded enolase was measured

TABLE 1. *E. coli* strains used in this study

Strain	Genotype	Source
BL21(DE3)	<i>F</i> ⁻ , <i>ompT</i> , <i>hsdS_B</i> (<i>r_B⁻m_B⁻) <i>gal</i>, <i>dcm</i> (DE3)</i>	Novagen
PET14	BL21(DE3) carrying pET14b (<i>amp</i>)	This study
TH-CPN60	BL21(DE3) carrying pET-CPN60 (<i>amp</i> , <i>cpn60</i>)	This study
TH-CPNS	BL21(DE3) carrying pET-CPNS (<i>amp</i> , <i>cpn10</i> , <i>cpn60</i>)	This study

as described below, and the refolding efficiency was evaluated as the specific activity of refolded enolase relative to that of untreated enolase.

Reactivation of thermally inactivated enolase Yeast enolase (Sigma) was thermally inactivated at 53°C for 30 min in 50 mM Tris-HCl (pH 7.5) containing 10 mM MgCl₂ and 20 mM KCl. Reactivation was examined at 30°C by diluting the inactivated enolase with 50 mM Tris-HCl (pH 7.5) containing 10 mM MgCl₂, 20 mM KCl and 2 mM DTT in the presence and absence of the reconstituted Tcnp60 tetradecamer of 0.4 μM. ATP was added to a final concentration of 2 mM. The final volume of the reaction mixture was 500 μl. The activity of the reactivated enolase was measured, and the reactivation efficiency was evaluated as the specific activity of reactivated enolase as a percentage of that of enolase before inactivation.

Measurement of enolase activity The activity of enolase was measured at 25°C using 2-phosphoglycerate (2-PGA) as the substrate (17). The enzyme solution (40 μl) was mixed with 0.96 ml of substrate solution (1 mM 2-PGA in 50 mM Tris-HCl [pH 7.5] and 1 mM MgCl₂). The activity was determined as the increase in absorbance at 240 nm as a function of time.

Growth of recombinant *E. coli* under high stress conditions

A single colony of *E. coli* PET14, TH-CPN60, or TH-CPNS was inoculated into 20 ml of LB medium containing 20 μg/ml of ampicillin at 37°C overnight. The pre-cultures were then diluted into 200 ml of LB broth containing 1 mM IPTG, 200 μg/ml ampicillin and 0.86 M NaCl and incubated at 37°C in shaking incubator. Cell growth was measured at OD₆₀₀.

Other methods Proteins were analyzed by polyacrylamide gel electrophoresis either in a 9% polyacrylamide gel containing 0.1% SDS or in a 6% polyacrylamide gel without SDS. Gels were stained by Coomassie Brilliant Blue (18). Protein concentration was determined by the method of Bradford (19) with bovine serum albumin as a standard.

RESULTS

Cloning and overexpression of Tcnp60 in *E. coli*

Tcnp60 was overexpressed in *E. coli* as His₆-tagged fusion proteins. SDS-PAGE analysis of the cell extract from *E. coli* TH-CPN60 showed an IPTG-induced major band at the 62-kDa position after IPTG induction which was not detected in the cell extracts of *E. coli* PET14. It was suggested that the 62-kDa band represented His₆-tagged Tcnp60 since its molecular size was in agreement with the molecular mass calculated from the deduced amino acid sequence of the His₆ fusion protein.

Purification and reconstitution of oligomeric recombinant Tcnp60 The fusion protein of Tcnp60 with the N-terminal His₆-tag was partially purified by nickel affinity chromatography. Since SDS-PAGE of the fraction purified by affinity chromatography showed some impurities, gel filtration column chromatography with TSK G4000SW_{XL} was used as an additional purification step and yielded the Tcnp60 protein showing a single band upon SDS-PAGE (Fig. 1A, inset). The retention time in the gel filtration chromatography revealed that the molecular size of the recombinant Tcnp60 corresponded to the monomer instead of the oligomer.

Reconstitution of the Tcnp60 oligomer was first attempted using Mg²⁺ and MgATP. However, a major shift in the apparent molecular size was not detected in the gel filtration chromatography analyses (Fig. 1A). Thus ammonium sul-

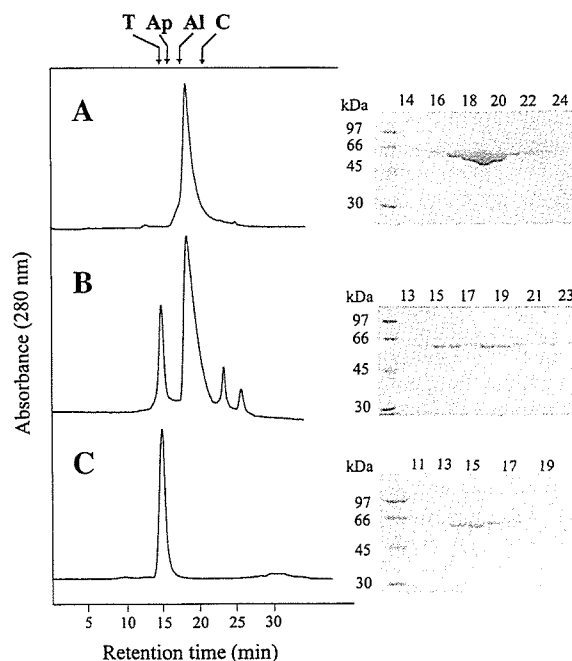


FIG. 1. Gel filtration chromatography profiles of the recombinant Tcnp60 upon TSK G4000SW_{XL} gel filtration chromatography followed by SDS-PAGE analysis of fractions (inset). (A) Purified recombinant Tcnp60 monomer. (B) Tcnp60 after incubation with MgATP and ammonium sulfate. (C) Rechromatography of the peak at 15 min in panel B. Protein elution was monitored by absorption at 280 nm. The arrows indicate the elution position of standard proteins: thyroglobulin (T, 669 kDa), apoferritin (Ap, 440 kDa), alcohol dehydrogenase (Al, 150 kDa) and carbonic anhydrase (C, 29 kDa). The numbers at the top of the SDS-polyacrylamide gels indicate the retention time (min) of fractions from the gel filtration chromatography.

fate was then added stepwise to the solution previously supplemented with MgCl₂ and ATP to a final concentration of 800 mM. Further additional ammonium sulfate was added to 60% saturation to precipitate the Tcnp60 protein. Then, the reconstituted Tcnp60 solution was applied onto a TSK G4000SW_{XL} gel filtration column. As shown in Fig. 1B, two distinct peaks were observed. The first peak was eluted before thyroglobulin, indicating that this protein was eluted as a large oligomeric complex, while a second peak corresponded to the original monomer. SDS-PAGE analysis revealed that both peaks contained a 62-kDa protein subunit of Tcnp60 (Fig. 1B, inset).

The first peak was collected and then concentrated to 6.4 μM by ultrafiltration. The apparent molecular size was determined to range from 800 to 880 kDa, by second round gel filtration chromatography (Fig. 1C) that is nearly identical to the theoretical molecular size of the tetradecamer. The protein band of these fractions contained a 62-kDa protein subunit of Tcnp60 (Fig. 1C, inset). The reconstituted oligomer was further analyzed by 6% native PAGE and migrated aberrantly slower than that of the monomeric fraction. This coincided with the result of gel filtration and suggested that the Tcnp60 oligomer was obtained.

Electron micrograph of the reconstituted Tcnp60 oligomer Electron microscopy of the reconstituted cpn60 oligomer revealed a number of particles with two types of

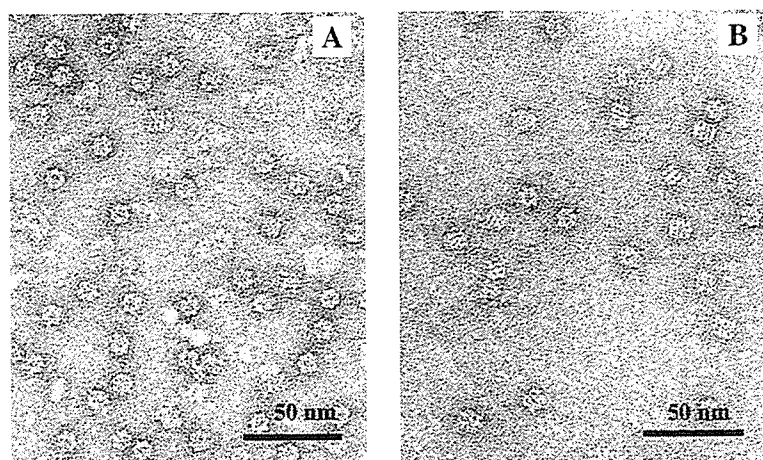


FIG. 2. Electron micrograph of the reconstituted Tcnp60 oligomer. (A) Top view of the Tcnp60 oligomer displaying the ring shape. (B) Side view of the Tcnp60 oligomer displaying the rectangular shape. The scale bar represents 50 nm.

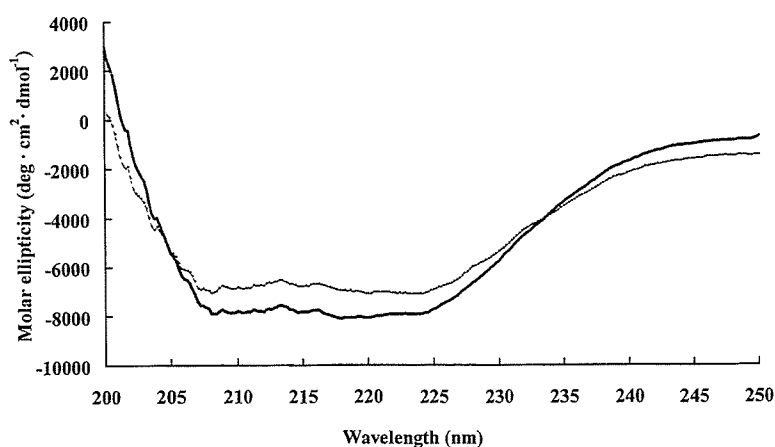


FIG. 3. The circular dichroism of the recombinant Tcnp60 tetradecamer (thick line) and monomer (thin line). The respective protein concentrations of the tetradecamer and monomer were 4.1 μM and 3.6 μM as protomer concentrations. The path length and the resolution were 0.1 cm and 0.2 nm, respectively. Spectra were measured four times and averaged.

forms showing toroidal (Fig. 2A) and rectangular structures (Fig. 2B). Careful observation revealed that the toroidal ring consisted of seven small subunits and the rectangle consisted of four stripes. These images correspond to typical top and side views of the chaperonin core complex, respectively, which represent the well-known double toroidal tetradecamer structure of natural chloroplast and bacterial cpn60.

Secondary structure of the Tcnp60 tetradecamer and monomer The secondary structure of the Tcnp60 tetradecamer and monomer was analyzed by circular dichroism (CD) spectroscopy in the far-UV from 200 to 250 nm (Fig. 3). Both CD spectra displayed the double minimum of the transitions at 208 and 222 nm, characteristics of α -helices. The helix contents of the tetradecamer and monomer estimated by the ellipticity were almost identical, suggesting no significant change in secondary structure during the *in vitro* reconstitution reaction.

ATPase activity of the Tcnp60 tetradecamer and monomer The ATPase activity of the reconstituted Tcnp60 tetradecamer was measured and compared with that of the

monomer (Fig. 4). The ATPase activity of the reconstituted oligomer was about eight times higher than that of the monomer, suggesting that Tcnp60 functioned in the tetradecameric form. No significant ATPase activity was observed in the absence of MgCl_2 , indicating that magnesium is essential for the ATPase activity. Thus, the subsequent experiments were performed using 10 mM MgCl_2 . The ATPase activity was measured under various concentrations of KCl and NaCl (Fig. 5). The activity increased markedly in the presence of KCl and remained stable up to 1 M, the highest concentration in this test. On the other hand, NaCl did not promote the ATPase activity, which decreased gradually with increasing NaCl concentration. The ATPase activity was also measured at various concentrations of NaCl and a constant KCl concentration (50 mM). As a result, the activity decreased with increasing NaCl concentration higher than 50 mM.

Refolding activity of the reconstituted Tcnp60 tetradecamer The refolding activity of the reconstituted Tcnp60 tetradecamer was analyzed using chemically and

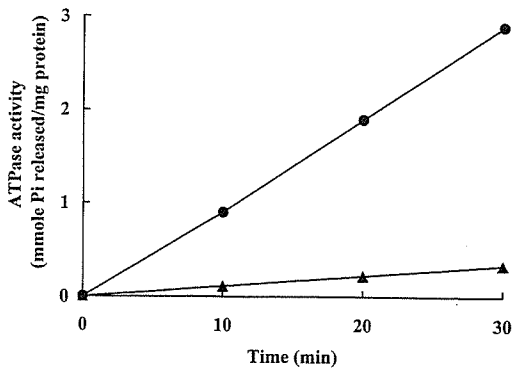


FIG. 4. ATPase activity of the recombinant Tcnp60 tetradecamer (circles) and monomer (triangles). The assay mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2 mM ATP, 50 mM KCl and 36 μ g of the purified Tcnp60 tetradecamer and monomer.

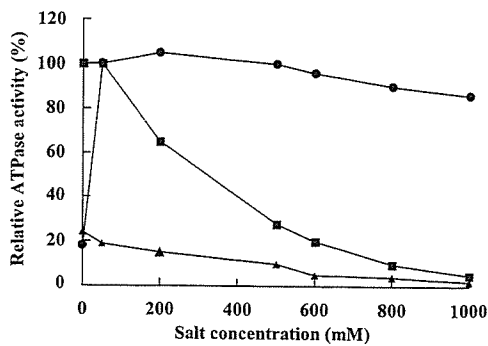


FIG. 5. ATPase activity of Tcnp60 under various salt concentrations. The Tcnp60 tetradecamer was supplemented with various salts as indicated below. The Tcnp60 tetradecamer was incubated in 50 mM Tris-HCl buffer pH 7.5 with 10 mM MgCl₂ with increasing KCl concentrations (circles), NaCl (triangles) and NaCl in the presence of 50 mM KCl (squares). ATPase activity at 10 mM MgCl₂ and 50 mM KCl was set to 100%.

thermally denatured enolases as substrates. The chemically denatured enolase spontaneously recovered up to about 60–70% of its initial activity. In the presence of ATP (2 mM) and the Tcnp60 tetradecamer (0.4 μ M), enolase activity was recovered to 86%, suggesting the refolding activity of the reconstituted Tcnp60 tetradecamer (Fig. 6). Incubation of enolase at 53°C for 30 min decreased its activity to less than 30%. Figure 7 shows the reactivation of thermally inactivated enolase by Tcnp60 in the presence of ATP. The thermally denatured enolase was gradually reactivated, while no recovery of its activity was observed in the absence of chaperonin. The refolding activity was measured under various concentrations of NaCl (Fig. 8). The activity decreased with increasing NaCl concentration but significant activity was detected at 1.2 M NaCl, the highest concentration in this experiment.

Effect of heterologous expression of Tcnp60 and Tcnp10 on growth under salt stress conditions The effect of the heterologous expression of Tcnp60 and Tcnp10 in *E. coli* on the growth under salt stress conditions was examined (Fig. 9). The growth of strain PET14 (vector control) exhibited a very long lag phase of about 12 h under 5%

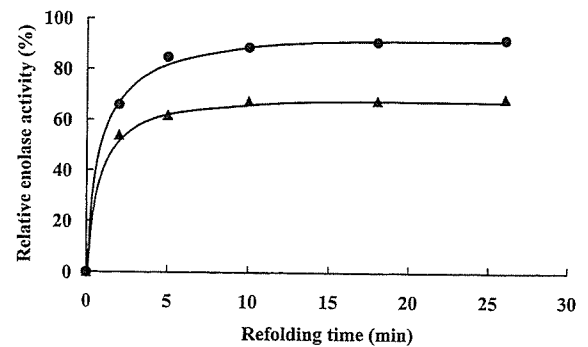


FIG. 6. Time course of refolding of Gdn-HCl-denatured enolase in the presence (circles) and absence (triangles) of the reconstituted Tcnp60 tetradecamer (0.4 μ M) and 2 mM ATP at 30°C. The activity of the refolded enolase was measured and the refolding efficiency was evaluated as the specific activity of refolded enolase relative to that of untreated enolase.

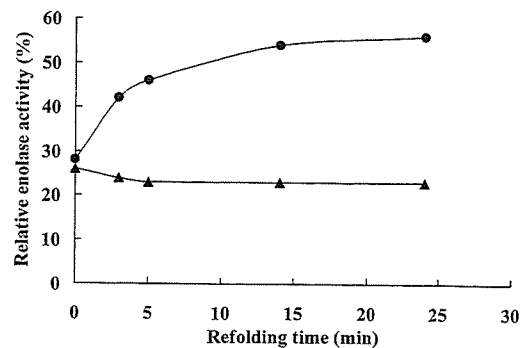


FIG. 7. Time course of refolding of thermally inactivated enolase in the presence (circles) and absence (triangles) of the reconstituted Tcnp60 tetradecamer (0.4 μ M) and 2 mM ATP at 30°C. The enzyme concentration was 0.2 μ M. The activity of the reactivated enolase was measured, and the reactivation efficiency was evaluated as the specific activity of reactivated enolase as a percentage of that of enolase before inactivation.

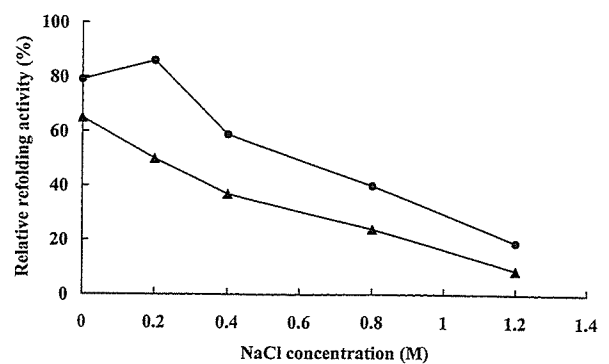


FIG. 8. The effect of various NaCl concentrations in the refolding of Gdn-HCl-denatured enolase with Tcnp60. Refolding was assayed 10 min after dilution as measured by the increasing absorbance at 240 nm, $\Delta A_{240}/\text{min}$ for 0.2 μ M of native enolase corresponding to 100% refolding. Results are expressed as the relative percentage of refolding activity. Refolding in the absence of chaperonin (triangles); refolding in the presence of reconstituted Tcnp60 and 2 mM ATP (circles).

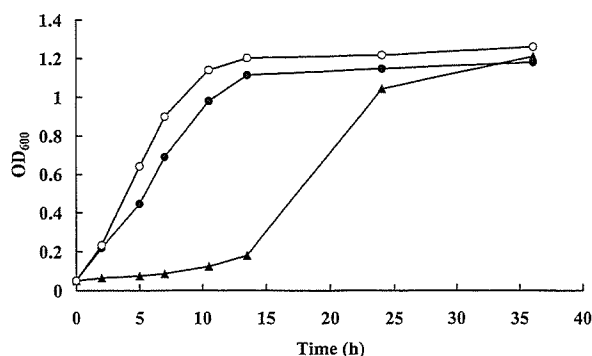


FIG. 9. Time course of cell growth of the recombinant *E. coli* PET14, TH-CPN60 and TH-CPNS in LB medium containing 5% (0.86 M) NaCl. Overnight cultures of the bacteria grown at 37°C in Luria-Bertani medium were diluted to an optical density at 600 nm (OD₆₀₀) of 0.05. Cultivation of strain TH-CPN60 (closed circles), TH-CPNS (open circles) and PET14 (triangles) was carried out in a shaking incubator at 37°C. Aliquots were removed at various time intervals to measure cell density (OD₆₀₀).

NaCl. As observed in *E. coli* TH-CPN60, expression of Tcnp60 shortened the prolonged lag-phase to the level of normal lag-phase observed under non-stress conditions. As observed in *E. coli* TH-CPNS, co-expression of Tcnp60 and Tcnp10 showed a higher promotive effect on the growth. These results suggested that the expression of Tcnp60 enabled the *E. coli* cells to rapidly adapt to the salt stress conditions and the efficiency was higher when expressed together with Tcnp10.

DISCUSSION

There is little available information about the oligomeric form of moderately halophilic cpn60 or lactic acid bacteria cpn60. There is only one report of the moderately halophilic *Pseudomonas* sp. #43 in which the purified GroEL was observed as various oligomeric forms which partially dissociated to the monomeric form during electrophoresis (20). In this study, the gel filtration chromatography analysis (Fig. 1A) and native PAGE analysis indicated that Tcnp60 expressed in *E. coli* existed in the monomeric form rather than the expected natural form of the tetradecamer. The CD spectrum showed that the Tcnp60 monomer was correctly folded as well as the reconstituted cpn60 oligomer (Fig. 3). However, the *E. coli*-expressed Tcnp60 could not form the oligomer spontaneously. This suggested that the lack of oligomerization was not due to misfolding of the monomer Tcnp60 subunit.

The lack of the oligomer may be due to the dissociation of the oligomer during the extraction or purification step as observed in *Pseudomonas* sp. #43 (20), mammalian mitochondrial chaperonin 60 (21), *Thermus thermophilus* HB8 (22), and *Thermoanaerobacter brockii* (23). At present, it is difficult to determine why the recombinant Tcnp60 oligomeric form was unstable and spontaneously dissociated into the monomeric form during the purification step. One possible reason for the dissociation of the Tcnp60 oligomer might be the release of nucleotides (15). It has been reported that the *in vitro* reconstitution of some cpn60s from various

microorganisms is achieved in the presence of MgATP or MgATP with ammonium sulfate (13–15). Based on these previous studies, we performed the reconstitution to obtain the functional oligomer in the same manner. After various attempts using reconstitution methods in which ammonium sulfate was added to the cpn60 solution containing MgATP with some modifications (14), we finally obtained the reconstituted Tcnp60 oligomer showing the well-known double toroidal, tetradecameric structure of the bacterial chaperonin core complex upon electron microscopy (Fig. 2). However, even after the reconstitution reaction, a major fraction of Tcnp60 still remained as the monomer as shown by the gel filtration (Fig. 1B), suggesting that another factor may be required to increase the yield of the oligomer. Structural cooperation with the co-chaperonin (Tcnp10) complex may also be important as found in the natural form of chaperonin complex.

The tetradecamer Tcnp60 complex showed much higher ATPase activity than the monomer (Fig. 4), although each subunit contains an ATP binding site. This agrees with some previous studies such as that on *E. coli* GroEL (13) or *Mycobacterium tuberculosis* GroEL (24) and suggests that the quaternary structure of cpn60 is very important for the ATPase activity. The effects of salts on the ATPase activity of the Tcnp60 tetradecamer were examined in detail (Fig. 5). Tcnp60 exhibited no ATPase activity in the absence of magnesium ions. The requirement for magnesium ions for the ATPase activity has been reported to involve coordination of the nucleotide as the cofactor to facilitate the hydrolysis of ATP by cpn60 (25). Potassium ions also markedly enhanced the ATPase activity of Tcnp60 and the activity was maintained up to 1 M potassium chloride, which the maximum concentration used in this experiment. On the contrary, the ATPase activity decreased with increasing sodium chloride concentration. Furthermore, in the presence of potassium ions, sodium ions showed an antagonistic effect on the activation of Tcnp60 ATPase activity by potassium ions. These halophilic properties of Tcnp60 ATPase activity may reflect a bioenergetic aspect of the halophile in which potassium ions are accumulated inside the cell instead of sodium ions (26). The inhibitory and antagonistic effects of sodium ions have been found for chaperonins from other halophiles, e.g., *Pseudomonas* sp. #43 (20) and *Haloflexax volcanii* (27).

Under low salt condition, the reconstituted Tcnp60 tetradecamer not only enhanced the refolding rate of the chemically denatured enolase, but also reactivated the thermally inactivated enolase to over 50% of its initial activity. In efforts to elucidate the mechanism by which the protein is refolded, significant differences between chemically and thermally denatured enolases have already been reported. It was suggested that the thermal denaturation process of enolase is irreversible, whereas the chemically denatured enolase is in dynamic and various unfolded states and has native secondary structure which can be disrupted (28–30). Our result is consistent with that observation. The activity of chemically denatured enolase used for this refolding assay spontaneously recovered from 0% to more than 60% of its initial activity within 5 min. On the other hand, heat-denatured enolase did not show any recovery of activity greater than 25% of the initial activity. These results imply that a

major fraction of the chemically denatured enolase was in the reversible state which can be refolded to the natural form, whereas the heat treatment changed the enolase molecule into the irreversibly-denatured form. The refolding of chemically denatured enolase by Tcnp60 was decreased significantly with increasing NaCl concentration. Another factor, *e.g.*, Tcnp10, may be required to function efficiently as a molecular chaperone under high salinity conditions.

The contribution of Tcnp60 to salinity adaptation was also observed *in vivo* by the heterologous expression system of the Tcnp60 gene in *E. coli* (Fig. 9). Overexpression of the Tcnp60 gene enabled the host cell to rapidly adapt to the salt stress condition and the effect was increased by co-expression with Tcnp10. This suggested that the *T. halophila* chaperonin proteins actually contribute to maintenance of the native state of various proteins in the cell growing under salt stress conditions. The same type of effect has been observed in *E. coli* transformed with the *T. halophila* molecular chaperone DnaK (5).

In order to gain a better understanding the role of the chaperonins in relation to salinity stress tolerance, further studies with the reconstituted Tcnp10 complex should be necessary. Substrate specificity of Tcnp60 would also be examined both *in vitro* and *in vivo*. This knowledge should shed light on the function of chaperonins in the moderately halophilic and halotolerant lactic acid bacteria.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Akemi Takade (Department of Bacteriology, Faculty of Medical Sciences, Graduate School, Kyushu University, Fukuoka) for the electron microscopy. This work was supported by the Mombusho Scholarship and JSPS Research Fellowships for young scientists.

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Survival and Death in Bacteria, 2005: 225-246 ISBN: 81-7736-236-4
Editor: Mamoru Yamada

13

Biofilm formation and entry into viable but nonculturable (VBNC) state: Survival strategy under stress conditions

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Abstract

In nature, bacteria are exposed to many different stressful conditions. Genetic and physiological adaptation of bacterial populations permits survival in environments with rapidly changing conditions. This review presents two aspects of bacterial cells under stressful conditions: biofilm formation and entry into viable but nonculturable (VBNC) state.

Introduction

In the natural environment, bacteria are constantly exposed to variable and stressful conditions. When

bacteria are transported from one environment to another, they are confronted by the changes in temperature, nutrient level, salinity, salt condition, pH, and many other factors (1). Many pathogenic and commensal bacteria are capable of transitioning between life in the environment and in the human host, and all must be able to adapt to sudden shifts in nutrient availability as well as to primary and secondary host immune defenses (2). Bacteria have evolved numerous adaptive mechanisms to facilitate their survival under changing environmental conditions. As a response to nutrient depletion, copiotrophic (3, 4), heterotrophic bacteria may undergo considerable morphological, physiological, and chemical changes (5-10). In fact, to survive energy- and nutrient-deprived conditions, non-spore-forming, heterotrophic bacteria are known to undergo an active adaptation program (10).

One such mechanism is phase variation, which occurs when the expression of a given factor is periodically altered such that it is either "on" or "off." These variations are generally spontaneous and reversible and occur at relatively high frequency ($>10^{-5}$ per generation) (11). Factors that are controlled by phase variation are often associated with the cell surface and include structures such as flagella, fimbriae, outer membrane proteins, and exo- and lipopolysaccharide (11). *Vibrio cholerae* can shift to a rugose colony morphology from its normal translucent smooth colony morphology in response to environmental stress including nutrient starvation (12, 13). The rugose strains produce more exopolysaccharide (EPS) than the smooth colony, acquired resistance to osmotic and oxidative stress, and promote biofilm formation (12-14). The similar phenomenon has been observed in *Vibrio parahaemolyticus* strains (15-17). Biofilm formation occurs on virtually any surface in any environment in which bacteria are present. These include natural (18) and industrial (19) ecosystems and infections (20-24). From an ecological standpoint, growth as a biofilm offers survival advantage in bacterial pathogenicity (20, 24-26).

The viable but nonculturable (VBNC) state is also thought to be a survival strategy adopted by bacteria when exposed to hostile environmental stress (1, 27-30). In the VBNC state, bacteria cannot be recovered by standard culture methods, but the cells still display active metabolism (31, 32), respiration (31), membrane integrity (33) and gene transcription with specific mRNA production (34).

In this review, we will discuss the bacterial survival strategy under stress conditions, focusing on biofilm formation of *V. cholerae* and the entry into VBNC state of several pathogenic bacteria.

Biofilm formation

Bacteria predominantly exist in sessile communities rather than as free-living cells. These communities develop as biofilms on all surfaces in aqueous