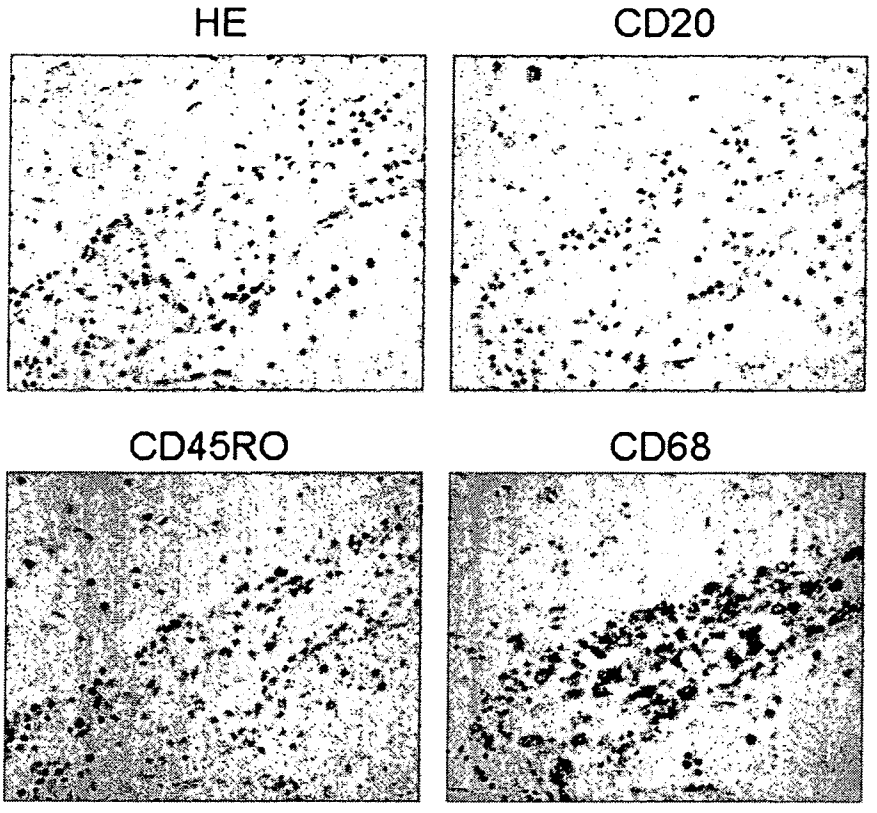

Fig. 5. Immunohistological examination of the infiltrated mononuclear cells in chronic progressive NB (autopsy). CD20 (L26), CD45RO (UCHL-1), CD68 (KP-1). Original magnification: ×50.

result from ischemia due to arterial occlusion. It was therefore suggested that repeated inflammatory attacks might be responsible for these lesions. Finally, there were still foci of mild infiltration of CD45RO+ T lymphocytes and CD68+ monocytes around small vessels (Fig. 9).

4. Discussion

It has been considered that CNS lesions in Behçet's disease are caused by vasculitis with venous predominance [8–10]. However, definite vasculitis is not so frequently observed [11]. Instead, most frequently observed abnormalities include small softening lesions with perivascular cellular infiltration of lymphocytes and sudanophilic foam-cells [6,8]. Consistently, in the present study, the lesions in acute NB showed marked perivascular cuffing of mononuclear cells around the small vessels. Of note, the results in the current studies have revealed that the mononuclear cells infiltrating around the small vessels were predominantly T lymphocytes and monocytes without B lymphocytes. More importantly, the neurons in the lesions

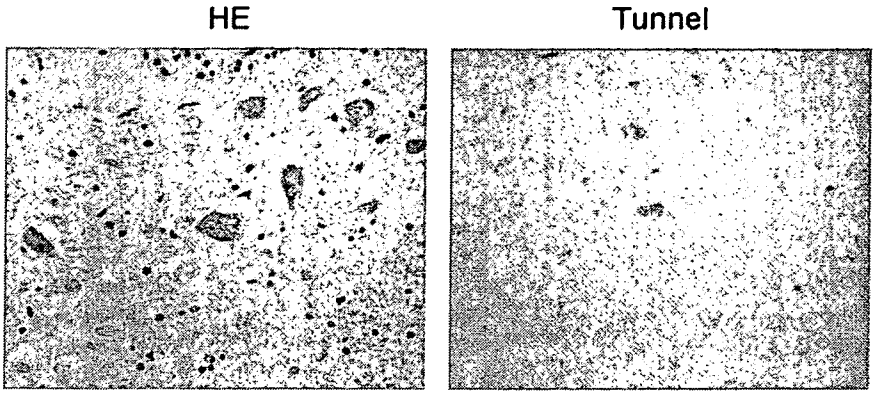

Fig. 6. TUNNEL staining of the mass lesion in chronic progressive NB (autopsy). Original magnification: ×25.

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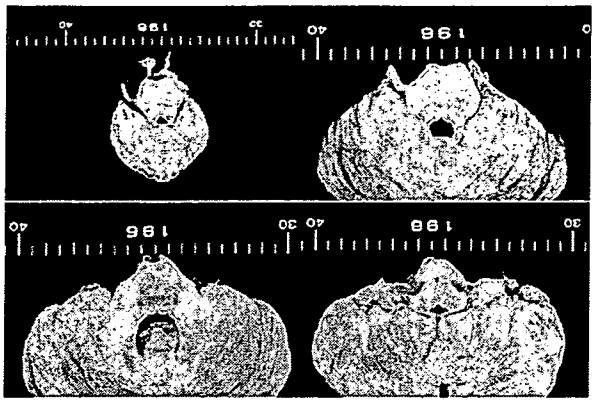

Fig. 7. Macroscopic findings of brainstem and cerebellum in NB in a long-term remission (autopsy).

were undergoing apoptosis. It is therefore suggested that soluble factors, including proinflammatory cytokines, produced by infiltrating T lymphocytes and monocytes might play a role in the induction of apoptosis of neurons. In this regard, we have disclosed that IL-6 levels were markedly elevated in cerebrospinal fluid from patients with NB [7]. Further studies to identify the precise mechanisms by which proinflammatory cytokines are produced within the CNS would be important for a complete understanding of the pathogenesis of NB.

We have recently disclosed that NB can be classified into acute NB and chronic progressive NB [7,12]. Thus, acute NB is characterized by acute meningoencephalitis, which responds well to corticosteroid and is usually self-limiting [7]. By contrast, chronic progressive NB is characterized by intractable, slowly progressive neuro-behavior changes and ataxia, along with persistent marked elevation of CSF IL-6 (usually more than 20 pg/ml) [1,6,7]. MRI findings may show only atrophy of brain stem and cerebellum, in contrast with the acute NB. The majority of patients with parenchymal CNS involvement had only single attacks, whereas one-third of patients underwent further attacks [3–5]. These patients correspond to acute NB in our classification [1]. Of note, previous studies also reported the presence of patients who underwent progressive deterioration leading to disability (primary or secondary progressive course) [3,4]. These patients are considered to be the same as chronic progressive NB in our classification [1]. These two types of NB are currently considered to represent different stages rather than be independent clinical entities [12]. In fact, most patients with chronic progressive NB had history of attacks of acute NB prior to the development of progressive neuropsychological symptoms [7]. Moreover, we have recently experienced some patients who displayed prolonged elevation of CSF IL-6 activity following acute NB [7]. Accordingly, the results in the current studies disclosed that the histopathological characteristics of chronic progressive NB are comparable to those of

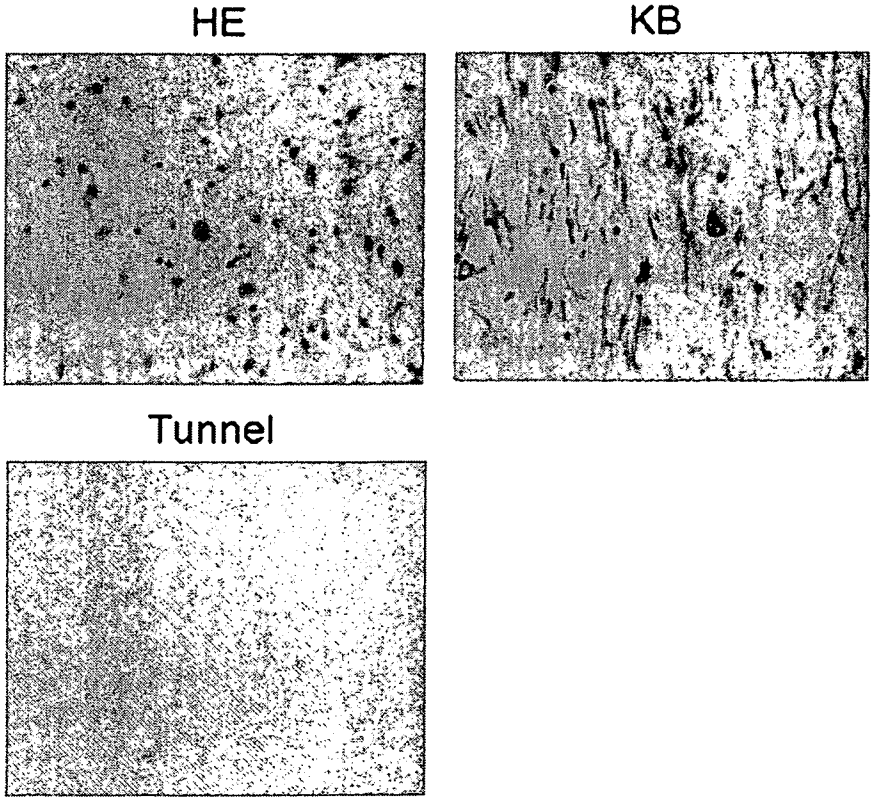

Fig. 8. Histopathology of the pontine lesion in NB in a long-term remission (autopsy). KB: Klüver-Barrera staining. Original magnification: ×25.

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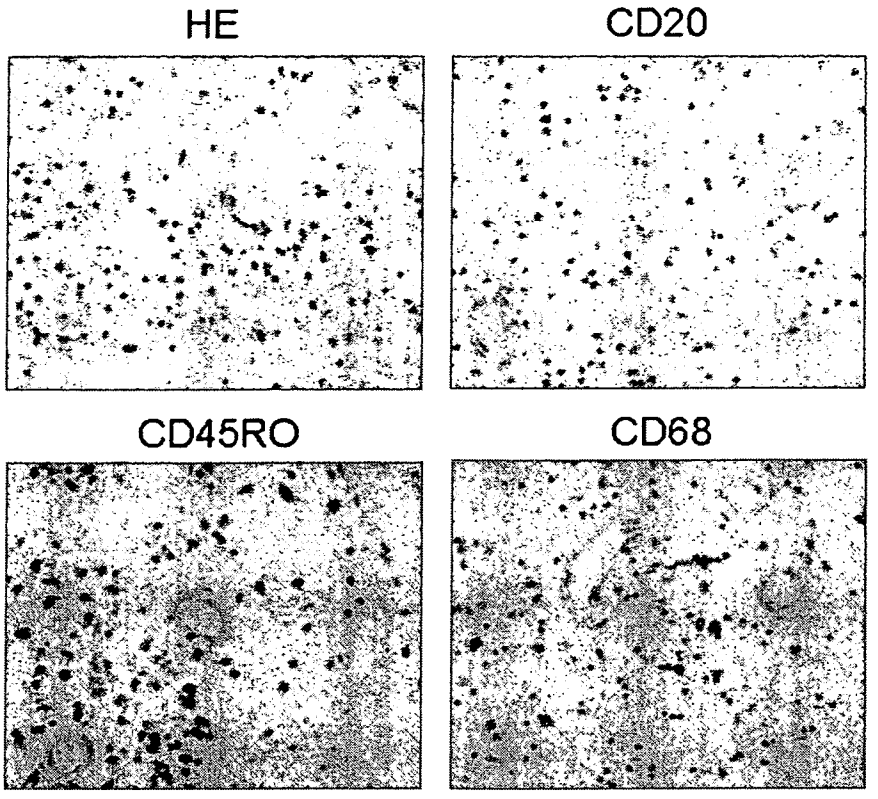

Fig. 9. Immunohistological examination of the infiltrated mononuclear cells in NB in a long-term remission (autopsy). CD20 (L26), CD45RO (UCHL-1), CD68 (KP-1). Original magnification: ×50.

acute NB. Thus, in the Patient 2 in the current studies, there were also foci of perivascular cuffing of mononuclear cells, consisting of T lymphocytes and activated monocytes/macrophages, with neurons undergoing apoptosis.

Previous studies revealed that binucleated neurons were often observed in the lesions of NB [9]. In the present study, binucleated neurons were also found in acute NB as well as in chronic progressive NB. More importantly, these binucleated neurons were positive for TUNNEL staining. It is therefore most likely that neurons undergoing apoptosis might form binucleated neurons. It has been shown that various factors cause apoptosis and binucleation in a variety of cells through the cytokinesis-blocking [13,14]. It is therefore suggested that some inflammatory assault might cause apoptosis and binucleation of neurons in NB. Further studies to explore the precise natures of such assault would be important.

Although the current studies showed that acute NB and chronic progressive NB have the common histopathological features, the mechanism of persistent inflammation in the CNS remains unclear. In this regard, it has been recently disclosed that HLA-B51 and cigarette smoking, and especially their combination, are risk factors for chronic progressive NB [15]. It is therefore likely that certain substances in cigarettes might be immunogenic in the context of HLA-B51, resulting in the persistent activation of immune responses within the CNS in Behçet's disease [15].

It is currently unclear which factors are responsible for the induction of apoptosis of neurons in NB. It has been shown that the concentration of IL-6 is markedly elevated in CSF from patients with acute NB and chronic progressive NB in relation to their disease activities [7]. Thus, CSF IL-6 was decreased when the disease activities were successfully suppressed [16]. Of note, previous studies also demonstrated that proinflammatory cytokines, including IL-6, play important roles in the damages of neurons [17–19]. Accordingly, there has been a growing appreciation of the destructive potential of elevated levels of IL-6 in the CNS. Thus, IL-6 has been found to cause neuronal degeneration and cell death in various disorders [20]. Moreover, recent studies have demonstrated that IL-6 mediates spinal cord neural injury through the signaling pathway from JAK/STAT activation to iNOS expression to poly (ADP-ribose) polymerase activation and cell death [21]. It is therefore most likely that high amounts of proinflammatory cytokines, especially IL-6, might be important for the induction of apoptosis of neurons in NB.

Perivascular infiltration of sudanophilic foam-cells has been described as one of the characteristic features of NB [8]. Accordingly, in the present study, perivascular cuffing of foam-cells was observed in acute NB as well as in chronic progressive NB. More importantly, it turned out that these cells were CD68+ cells. It is therefore suggested that these

foam-cells might be activated macrophages, which phagocyte damaged white matters (myelin).

The long-term course of NB after remission has not been explored. Patient 3 in the current studies represents a patient of NB who had been in a long-term remission after successful treatment. However, there still remained foci of perivascular cuffing of T lymphocytes and monocytes in this patient, suggesting that small inflammatory attacks still took place in NB during the remission phase.

Previous studies revealed that the most frequent CNS finding in NB was a preference for involvement of brainstem-diencephalon and pontobulbar region [2]. Koçer et al suggested that the anatomic variability of venous anatomic arrangements at different levels of the CNS might explain such predilection [10]. Consistently, in Patient 3 of our study with a long-term course of NB showed atrophy of brainstem and cerebellum, as has been reported previously [2]. On the other hand, arterial involvement resulting in CNS vascular disease is rare in Behçet's disease [6]. Of note, the reservation of viable neurons within the parenchymal lesions with isomorphic gliosis in our patient with a long course of NB obviates the possibility that the lesions might result from ischemia due to vasculitis of arteries. Rather, these findings suggest that relapsing-remitting small attacks with perivascular cuffing of mononuclear cells might be repeated during the long course in this patient.

In summary, the current studies have disclosed the characteristics of histopathology in 3 patients with different phases of NB, including acute phase, chronic progressive phase, and remission phase. The common features throughout the courses of NB appeared to be relapsing-remitting attacks of inflammation with perivascular infiltration of T lymphocytes and activated monocytes/macrophages. The magnitude and the duration of the inflammation might determine the clinical features and outcome. Finally, it is suggested that the production of huge amounts of proinflammatory cytokines, such as IL-6, might induce neuronal dysfunction and apoptosis. Further studies to delineate the triggers for the inflammation attacks as well as the factors for their perpetuation would be important for a complete understanding of the pathogenesis of NB.

Acknowledgment

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References

[1] Hirohata S, Kikuchi H. Behçet's disease. Arthritis Res Ther 2003;5:139–46.
[2] Serdaroglu P. Behçet's disease and the nervous system. J Neurol 1998;245:197–205.
[3] Kidd D, Steuer A, Denman AM, Rudge P. Neurological complications in Behçet's syndrome. Brain 1999;122:2183–94.
[4] Akman-Demir G, Serdaroglu P, Tasçi B, The Neuro-Behçet Study Group. Clinical patterns of neurological involvement in Behçet's disease: evaluation of 200 patients. Brain 1999;122:2171–82.
[5] Siva A, Kantarci OH, Saip S, Altintas A, Hamuryudan V, Islak C, et al. Behçet's disease: diagnostic and prognostic aspects of neurological involvement. J Neurol 2001;248:95–103.
[6] Siva A, Altintas A, Saip S. Behçet's syndrome and the nervous system. Curr Opin Neurol 2004;17:347–57.
[7] Hirohata S, Isshi K, Oguchi H, Ohse T, Haraoka H, Takeuchi A, et al. Cerebrospinal fluid interleukin-6 in progressive neuro-Behcet's syndrome. Clin Immunol Immunopathol 1997;82:12–7.
[8] McMenemey WH, Lawrence BJ. Encephalomyelopathy in Behçet's disease: report of necropsy findings in two cases. Lancet 1957;24: 353–8.
[9] Kawakita H, Nishimura M, Satoh Y, Shibata N. Neurological aspects of Behçet's disease: a case report and clinico-pathological review of the literature in Japan. J Neurol Sci 1967;5:417–38.
[10] Koçer N, Islak C, Siva A, Saip S, Akman C, Kantarci O, et al. CNS involvement in neuro-Behçet's syndrome: an MR study. Am J Neuroradiol 1999;20:1015–24.
[11] Hadfield MG, Aydin F, Lippman HR, Sanders KM. Neuro-Behçet's disease. Clin Neuropathol 1997;16:55–60.
[12] Kawai M, Hirohata S. Cerebrospinal fluid beta(2)-microglobulin in neuro-Behcet's syndrome. J Neurol Sci 2000;179:132–9.
[13] McKelvie PA, Daniell M. Impression cytology following mitomycin C therapy for ocular surface squamous neoplasia. Br J Ophthalmol 2001;85: 1115–9.
[14] Picerno I, Chirico C, Condello S, Visalli G, Ferlazzo N, Gorgone G, et al. Homocysteine induces DNA damage and alterations in proliferative capacity of T-lymphocytes: a model for immunosenescence. Biogerontology 2007;8:111–9.
[15] Aramaki K, Kikuchi H, Hirohata S. HLA-B51 and cigarette smoking as risk factors for chronic progressive neurological manifestations in Behcet's disease. Mod Rheumatol 2007;17:81–2.
[16] Hirohata S, Suda H, Hashimoto T. Low-dose weekly methotrexate for progressive neuropsychiatric manifestations in Behçet's disease. J Neurol Sci 1998;159:181–5.
[17] Kessler JA, Ludlam WH, Freidin MM, Hall DH, Michaelson MD, Spray DC, et al. Cytokine-induced programmed death of cultured sympathetic neurons. Neuron 1993;11:1123–32.
[18] Heyser CJ, Masliah E, Samimi A, Campbell IL, Gold LH. Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. Proc Natl Acad Sci U S A 1997;94:1500–5.
[19] Gruol DL, Nelson TE. Purkinje neuron physiology is altered by the inflammatory factor interlukin-6. Cerebellum 2005;4:198–205.
[20] Gadient RA, Otten UH. Interleukin-6 (IL-6)—a molecule with both beneficial and destructive potentials. Prog Neurobiol 1997;52:379–90.
[21] Kaplin AI, Deshpande DM, Scott E, Krishnan C, Carmen JS, Shats I, et al. IL-6 induces regionally selective spinal cord injury in patients with the neuroinflammatory disorder transverse myelitis. J Clin Invest 2005;115: 2731–41.

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REVIEW ARTICLE

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Laser-mediated microdissection for analysis of gene expression in synovial tissue

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Abstract In experimental rheumatology, *transcriptomics* is one of the most important methods for investigating the pathogenesis of diseases. The biological material of most studies on rheumatoid arthritis has been bulk rheumatoid synovial tissues, but they are not suitable because they consist of several kinds of cells or structures. Laser-mediated microdissection (LMM) is a useful tool for isolating particular cells from tissue specimen to assess the functions of each cell. The LMM system employs a combination of a microscope and a laser-beam generator to cut out target areas on cryosections. Tissue compartments or even a single viable cell can be isolated using a non-focused laser beam without direct contact to avoid contamination, and this process is called laser pressure catapulting. An ultraviolet-A laser enables target cells to be procured without any influence on the surrounding. This technique has already been used in several studies in rheumatology, and its validity has been confirmed. Combined with other new techniques such as real-time quantitative polymerase chain reaction or microarray analysis, LMM is becoming more important in the analysis of gene expression in rheumatology.

Key words Laser capture microdissection · Laser-mediated microdissection · Rheumatoid arthritis

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Introduction

The mainstream of current medical research is moving toward proteomics based on genomics. However, gene analysis is still a substantial part of research, not only with regard to the pathogenesis of diseases but also with regard to the normal development and physiology of cells and tissues. Various methods have been employed for such studies, but analysis has mainly been performed with bulk tissue samples or bulk-cultured cells consisting of heterogeneous populations in spite of several problems. First, bulk tissue contains a variety of cells and only some of them are relevant to any particular study. When performing gene expression analysis to investigate the pathogenesis of diseases, pure samples from the target lesion are essential and a credible result cannot be obtained from bulk tissue samples because of contamination by normal cells. For example, rheumatoid synovium contains several cellular components, such as fibroblasts, macrophages, and lymphocytes, and is composed structurally of a lining layer, a sublining, vessels, and lymphoid follicles, in which cells show various differences in gene expression. Gene expression in each tissue compartment should be analyzed separately for understanding the contribution made by each component of a tissue to the pathogenesis of rheumatoid arthritis (RA).

Second, there are many kinds of cells which are difficult to isolate, to obtain in a differentiated state, or to culture in an artificial environment. Moreover, cells cultured from organs or tissues can alter the genetic profile and other features in response to environmental changes. Therefore, it is necessary to procure particular parts of a tissue or individual target cells for reliable genetic analysis. Laser-mediated microdissection (LMM), also known as laser microdissection (LMD), is a technique that was developed for this purpose, allowing specific cells to be cut out of a tissue easily for analysis with minimal loss of proteins and DNA or RNA.[1] The technique is also called laser capture microdissection (LCM) or laser microbeam microdissection coupled with laser pressure catapulting (LMM/LPC),

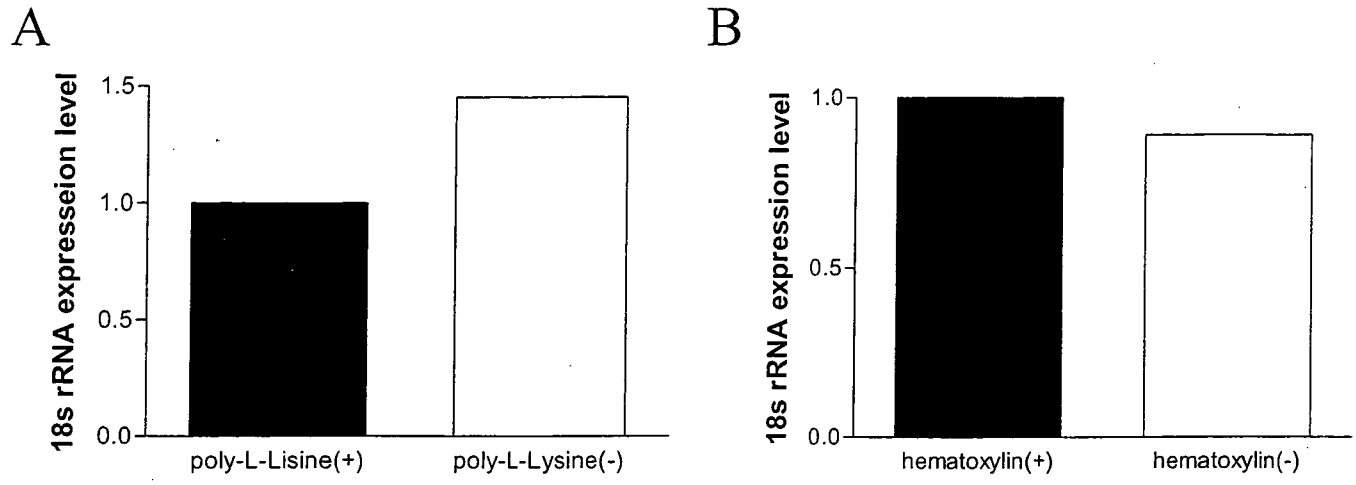

Fig. 1. The amounts of RNA of housekeeping gene (18s rRNA) from the microdissected samples with or without poly-L-lysine coated slides were measured with real-time quantitative polymerase chain reaction (PCR). The same vessels in serial sections of synovial tissues of patients with rheumatoid arthritis (RA) on coated or non-coated slides were obtained with laser-mediated microdissection (LMM). The total RNA of the vessels was extracted and the cDNA was synthesized, which was applied to real-time quantitative PCR analysis using the Lightcycler system and SYBR Green detection. Poly-L-lysine coating of slides may reduce the total RNA obtained from microdissected samples (**a**). Similarly, the effect of hematoxylin staining was analyzed. The reduction caused by hematoxylin staining was not detected (**b**). The relative mRNA levels were normalized against the values of samples with the process of poly-L-lysine (**a**) or hematoxylin (**b**), which were set as 1

especially when the system allows the isolation of specific cells dissected from a tissue sample on a slide. Before this technique was developed, cells had to be extracted manually.[2]

The LMM technique has already been widely applied to various fields of medical research such as oncology,[3,4] nephrology,[5] endocrinology,[6] dermatology,[7] and rheumatology, as described in this article, and it facilitates the analysis of gene expression in every field of research.

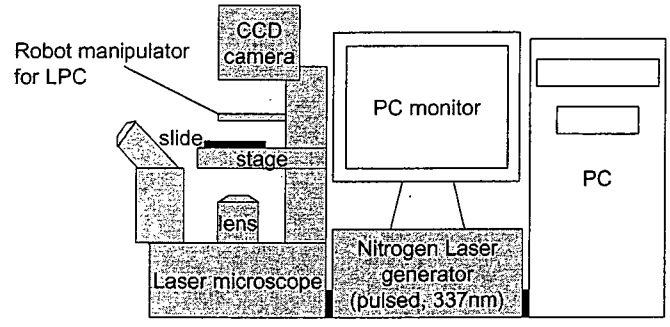

Fig. 2. The LMM system consists of a laser microscope, a laser generator, and a PC with a monitor. Target regions in a tissue on a slide are viewed and marked on the PC monitor through a charge-coupled device camera. A laser beam is generated in the generator and goes upward through the lens, penetrating and cutting the tissue along the outlines of marked areas automatically with the moving stage

LMM technique

Frozen tissue samples (especially, synovial tissues from patients with RA) have been generally analyzed using LMM in many rheumatological experiments, although a single viable cell can be removed from a mixed culture.[8] Today there are various LMM systems, such as that produced by Arcturus Engineering (Mountain View, CA, USA) or that from Leica Microsystems (Bensheim, Germany). The former system was developed from the original LCM system invented by the National Cancer Institute of the National Institutes of Health (Bethesda, MD, USA), and it detaches target cells from a tissue sample by focally melting the polymer membrane on which the tissue is mounted.[1] The latter is the only system that employs an upright microscope, and target cells are extracted into a tube set below the stage utilizing gravity without contact.[9] Our group has also employed the use of the system by P.A.L.M. Microlaser Technologies (Wolfratshausen, Germany).[10] In brief, fresh tissue samples are embedded in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. Cryosections (5–8μm thick) are made using a cryostat. The sections are mounted on glass slides coated with a polyethylene naphthalate (PEN) film. To facilitate the attachment of cryosections to the slide, the PEN film can be coated with 0.1% poly-L-lysine which causes ionic charge, but this process can reduce the yield of RNA (Fig. 1a). Glass slides coated with PEN films are available from several companies. The film on the slide allows a microdissected area to be peeled off without disintegration of the cells. The slides are fixed in 5% acetic acid/95% ethanol or in other solutions such as 75% ethanol or 100% methanol. If necessary, the slides can be stained with stains such as hematoxylin, toluidine blue, or the HistoGene Staining Kit (Arcturus) after fixation for detecting particular cells prior to microdissection.[11,12] The loss of RNA due to staining with hematoxylin is negligible (Fig. 1b). Even immunohistochemical staining can be performed to distinguish particular cells for mRNA analysis using LMM.[13,14] The slides should be used for analysis as soon as possible, or stored at –80°C until use to avoid the loss of RNA.

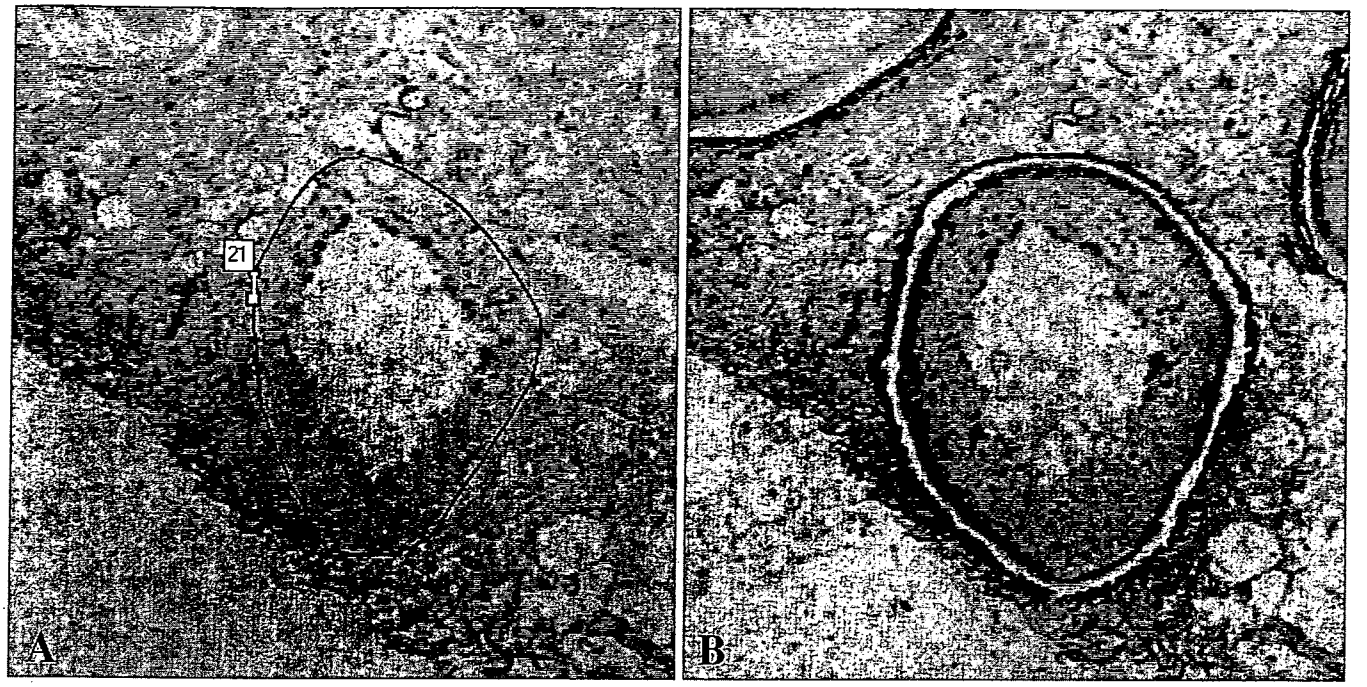

Fig. 3. A cryosection of synovial tissue of RA is mounted on a slide with a membrane film and set on the LMM microscope. The sample is observed on the PC monitor and a target area is marked (**a**). Afterward, the defined line is cut by a laser beam (**b**) (×100)

The P.A.L.M. MicroLaser system consists of a laser microscope with a charge-coupled device camera, a laser generator, and a computer (PC) with monitor for general control and operation (Fig. 2). A slide is set on the stage of the laser microscope and target regions are viewed on the PC monitor, marked by an operator and cut out by a laser beam as the stage is moved automatically (Fig. 3). Recently, infrared lasers which cause thermal tissue damage have been replaced by pulsed ultraviolet (UV)-A lasers in most LMM systems because the UV-A laser does not cut with heat transformation but with a photochemical process. The phenomenon of ablative photo-decomposition occurs only at the focal point of the laser, and the surrounding tissues or cells remain completely intact. Therefore, this process is called cold ablation.[15,16]

Microdissected cells are lifted up vertically by a non-focused laser beam and are collected in a tube by a robot manipulator (Fig. 4). This method is called laser pressure catapulting and it is a useful technique that enables non-contact preparation of samples with an LMM system, which is important to avoid contamination. However, larger pieces of tissue can also be collected by hand with a 27-gauge needle after microdissection. Following collection of the desired samples, the extraction of DNA, RNA, or protein can be performed.

RNA is extracted for subsequent analysis of gene expression using techniques such as real-time quantitative polymerase chain reaction (PCR), differential display, and microarray analysis. Unlike the conventional PCR method, the relative or absolute amount of RNA can be accurately and easily measured using real-time quantitative PCR.[17] One of the most popular real-time quantitative PCR methods employs SYBR Green fluorescent dye. The quantity of

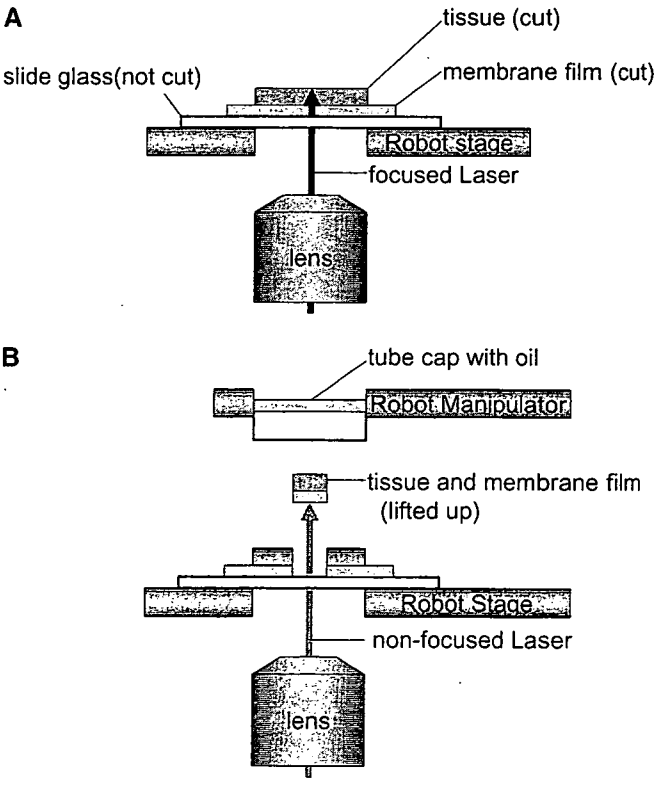

Fig. 4. Side view. A focused laser beam, which comes through a lens under the robot stage, does not cut a glass slide but does cut a transparent membrane film and a tissue with "cold ablation" (**a**). Microdissected cells with a membrane film are lifted up vertically by a non-focused laser and collected in a tube cap which is sighted and fixed upside down with a robot manipulator. The lifted sample is caught with oil in the bottom of the cap. This system is called laser pressure catapulting (**b**)

the PCR product is measured during every cycle by detecting the fluorescence which is generated when SYBR Green binds to double-stranded DNA synthesized during the PCR reaction.[18,19] Differential display, also called fingerprinting, is a technique to detect the differential expression of mRNA employing processes such as arbitrary primed PCR and gel electrophoresis.[20] Microarray analysis is a recently developed tool for the assessment of gene expression, and its high throughput facilitates gene expression profiling.[21] In brief, a microarray is composed of a glass slide, a plastic chip, or a nylon membrane on which single-stranded cDNA clones of various genes or a large set of oligonucleotides are fixed in a high-density array. The mRNA expression in a sample is detected with the binding of labeled single-stranded cDNA synthesized from sample mRNA to complementary sequences on the microarray.

The number of cells necessary for gene analysis depends on various factors such as the level of gene expression, the type of cells, treatment of sample, and the methods of isolating, amplifying, and analyzing mRNA. For example, Judex et al.[22] reported that 600 cells obtained with LMM from the synovial lining and sublining of RA patients provided sufficient mRNA to carry out differential display analysis without mRNA amplification, whereas our group needed 5000–8000 cells from the synovial vessels of patients with RA or osteoarthritis (OA) to quantify mRNA for every target gene accurately by real-time quantitative PCR.[23] In contrast, only 10–20 macrophages, dendritic cells, or T cells from RA synovium with immunohistochemical staining provided enough material for real-time polymerase chain reaction (RT-PCR).[24]

A critical weak point of LMM is its low yield of biological starting materials, especially RNA, because sample cells were obtained from a limited area of thin cryosections (5–8μm thick) and every preparative procedure such as fixation or staining can degrade RNA. Then some methods were invented to amplify RNA especially for microarray assays. The switch mechanism at the 5′ end of RNA templates (SMART) with reverse transcription is a common protocol. Another protocol employs a bacterial RNA polymerase promoter sequence such as the T7 RNA polymerase, with which single-cell gene expression profiling is possible.[25–28]

Application of LMM for rheumatology

Laser-mediated microdissection has been applied thus far in the field of rheumatology. Judex et al.[22] demonstrated the differential expressions of fibronectin 1, ciz-1, and thrombospondin 4 in cells between the lining layer and the sublining in RA synovium with LMM followed by nested RNA arbitrarily primed-PCR and differential display, and the result was confirmed by in situ hybridization at the mRNA level and immunohistochemical staining at the protein level. This is the first report of applying the LMM technique for a study in rheumatology. These procedures were also detailed in another report.[29]

Fractalkine (neurotactin, CX3CL1) is a chemokine thought to be concerned with monocyte chemotaxis and angiogenesis in the rheumatoid synovium. Fractalkine receptor (CX3CR1) was found in different cell types of RA synovium.[24] Each type of cells such as macrophages (CD68+), dendritic cells (CD1a+), and T cells (CD3+) was individually distinguished with immunohistochemical staining and thereafter microdissected and collected separately. Thus, using LMM, fractalkine receptor mRNA expression in each type of cells in RA synovium was detected with RT-PCR.

Currently, the principal manner for the analysis of gene expression profiling in rheumatology is to procure pure groups of cells from rheumatoid synovial tissue and analyze them with microarrays. Whereas whole synovial tissues of RA have been used for analysis with microarrays in several studies, Tsubaki et al.[30] applied LMM to obtain the synovial lining tissues in early RA. In this study, samples were clustered into two groups on the basis of their gene expression profile and the grouping correlated with the histological evaluation of each sample. The different expression profiles of several candidate genes between these two groups suggested differences in the pathogenesis of synovitis and could be employed for diagnostic and prognostic studies of early RA. In another study, Tsubaki et al.[31] procured cells from the synovial lining, sublining, vascular, and lymphoid follicular regions separately in RA synovial tissues and analyzed mRNA expression of a chemokine receptor CXCR3 in each region using RT-PCR. The mRNA expression was confirmed with immunohistochemical staining at the protein level, and the result suggested that plasma cells expressing CXCR3 in early RA synovium is recruited via the ligand, Mig/CXCL9, which is produced by fibroblasts mainly in synovial sublining regions. It is notable that they picked several distinct regions individually from a cryosection and detected mRNA expression in each region because this style of analysis could not be carried out without the LMM technique.

Recently, angiogenesis has been recognized as a crucial factor to develop and perpetuate rheumatoid synovitis.[32] Our group directed our attention to angiogenesis in RA synovium for the understanding of pathogenesis of RA.[23] We analyzed the expressions of several genes including those concerned with angiogenesis in synovial vessels of RA and OA which were procured with LMM. Of the seven genes analyzed with real-time quantitative PCR, two genes showed a significant differential expression between RA and OA synovial vessels. Id2, the inhibitor of differentiation and promoter of proliferation, was highly expressed in OA vessels than that of RA. It was confirmed with real-time quantitative PCR at the mRNA level (Fig. 5) and with immunohistochemistry at the protein level (Fig. 6). In contrast, the expression of vascular endothelial growth factor receptor-1 (VEGFR-1; Flt-1) was higher in RA vessels than in OA. These results were supported by immunohistochemical staining, and it was exhibited that small synovial vessels in synovial tissue can be a material of mRNA analysis.

Laser-mediated microdissection is also applied for the analysis of diseases other than RA. Plasma cells in synovial

lesions of patients with Lyme disease were identified with immunofluorescent staining using an anti-CD138 antibody, procured with LMM, and the mRNA was analyzed with RT-PCR.[14] In this study, fast and careful procedures of fixation, immunofluorescent staining, and development were adopted to prepare LMM samples, especially for the avoidance of degradation of RNA. In brief, the sample slides were fixed in acetone at 4°C for 4 min, exposed to primary and secondary antibody for 10 min and 5 min on a cold block, respectively, followed by procurement of target cells with LMM.

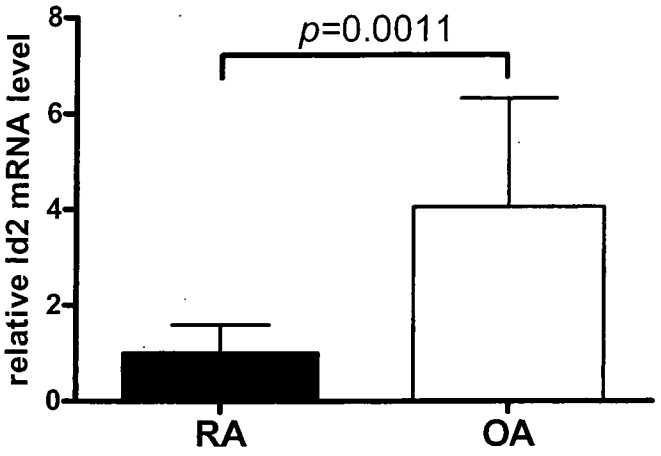

Fig. 5. Relative mRNA levels of Id2 in synovial vessels of RA or osteoarthritis (OA). The Id2 mRNA level of synovial vessels of OA was fourfold higher than that of RA. Values are normalized with endogenous control (18s rRNA) and the mean value of relative mRNA level of RA is set as baseline (reference value = 1) Source: Hashimoto et al.[23]

Immunoglobulin V regions expressed in plasma cells of Lyme arthritis were amplified with RT-PCR, and the analysis of nucleotide sequence illustrated the repertoire and mutational status of antibodies, suggesting the pathogenesis of chronic arthritis through the potential cross activity of antigens.

Conclusion

Although the LMM technique was originally invented and developed mainly in the field of oncology, it has been popularized into a variety of fields in medical science, and the instrumentations and procedures used in LMM have progressed over the years. Despite the cost, it is certain that the LMM technique is necessary to develop new dimensions of research, as mentioned above, not only in the field of rheumatology. Although the LMM technique has been utilized mainly for the analysis of synovial tissues in rheumatology research, currently, a single cell or living cells can also be isolated with the LMM technique. Not only RNA or DNA but also proteins can be analyzed, especially for the investigation of proteomics, which can reveal the functions of each cell in the disease. On the technical side, better preparative methods, including staining without loss of yield, should be established. The procedures for cutting and capturing cells should be further automated to save the work of operators and to obtain materials accurately and objectively. Future studies of gene expression analysis need technologies for obtaining enough nucleic acid from limited amount of material and high-throughput precise assays such as microarrays; LMM plays an important role in this.

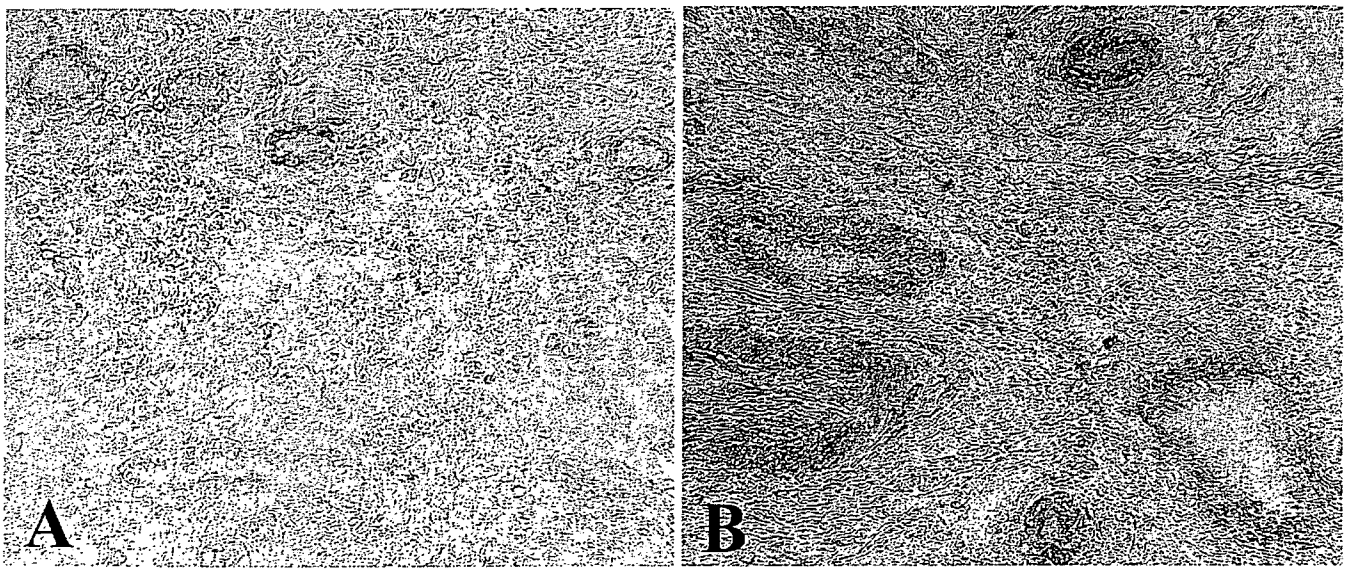

Fig. 6. Id2 protein expressed in synovial tissues of RA or OA detected with immunohistochemistry using a specific antibody. Strong positive staining of Id2 was seen in synovial vessels of OA (**b**) compared with those of RA (**a**). These stainings of Id2 corresponded to the levels of Id2 mRNA expression in synovial vessels of RA and OA (×100)

References

1. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, et al. Laser capture microdissection. Science 1996;274:998–1001.
2. Maitra A, Wistuba II, Gazdar AF. Microdissection and the study of cancer pathways. Curr Mol Med 2001;1:153–62.
3. Cör A, Vogt N, Malfoy B. Microdissection techniques for cancer analysis. Folia Biol (Praha) 2002;48:3–8.
4. Lechner S, Müller-Ladner U, Renke B, Scholmerich J, Ruschoff J, Kullmann F. Gene expression pattern of laser microdissected colonic crypts of adenomas with low grade dysplasia. Gut 2003;52: 1148–53.
5. Fries JW, Roth T, Dienes HP, Weber M, Odenthal M. A novel evaluation method for paraffinized human renal biopsies using quantitative analysis of microdissected glomeruli and VCAM-1 as marker of inflammatory mesangial cell activation. Nephrol Dial Transplant 2003;18:710–6.
6. Ho Hong S, Young Nah H, Yoon Lee J, Chan Gye M, Hoon Kim C, Kyoo Kim M. Analysis of estrogen-regulated genes in mouse uterus using cDNA microarray and laser capture microdissection. J Endocrinol 2004;181:157–67.
7. Yazdi AS, Puchta U, Flaig MJ, Sander CA. Laser-capture microdissection: applications in routine molecular dermatopathology. J Cutan Pathol 2004;31:465–70.
8. Burgemeister R. New aspects of laser microdissection in research and routine. J Histochem Cytochem 2005;53:409–12.
9. Kolble K. The LEICA microdissection system: design and applications. J Mol Med 2000;78B:24–5.
10. Burgemeister R, Gangnus R, Haar B, Schutze K, Sauer U. High quality RNA retrieved from samples obtained by using LMPC (laser microdissection and pressure catapulting) technology. Pathol Res Pract 2003;199:431–6.
11. Tachikawa T, Irie T. A new molecular biology approach in morphology: basic method and application of laser microdissection. Med Electron Microsc 2004;37:82–8.
12. Mikulowska-Mennis A, Taylor TB, Vishnu P, Michie SA, Raja R, Horner N, et al. High-quality RNA from cells isolated by laser capture microdissection. Biotechniques 2002;33:176–9.
13. Fend F, Emmert-Buck MR, Chuaqui R, Cole K, Lee J, Liotta L, et al. Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. Am J Pathol 1999; 154:61–6.
14. Ghosh S, Steere AC, Stollar BD, Huber BT. In situ diversification of the antibody repertoire in chronic Lyme arthritis synovium. J Immunol 2005;174:2860–9.
15. Srinivasan R. Ablation of polymers and biological tissue by ultraviolet lasers. Science 1986;234:559–65.
16. Vogel A, Venugopalan V. Mechanisms of pulsed laser ablation of biological tissues. Chem Rev 2003;103:577–644.
17. Orlando C, Pinzani P, Pazzagli M. Developments in quantitative PCR. Clin Chem Lab Med 1998;36:255–69.
18. Wittwer CT, Ririe KM, Andrei RV, David DA, Gundry RA, Balis UJ. The Lightcycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 1997;22:176–81.
19. Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 1998;24:954–8, 960, 962.
20. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 1992;257: 967–71.
21. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995;270:467–70.
22. Judex M, Neumann E, Lechner S, Dietmaier W, Ballhorn W, Gifka J, et al. Laser-mediated microdissection facilitates analysis of area-specific gene expression in rheumatoid synovium. Arthritis Rheum 2003;48:97–102.
23. Hashimoto A, Tarner IH, Bohle RM, Gaumann A, Manetti M, Distler O, et al. Analysis of vascular gene expression in arthritic synovium by laser-mediated microdissection. Arthritis Rheum 2007;56:1094–105.
24. Blaschke S, Koziolek M, Schwarz A, Benöhr P, Middel P, Schwarz G, et al. Proinflammatory role of fractalkine (CX3CL1) in rheumatoid arthritis. J Rheum 2003;30:1918–27.
25. Neumann E, Kullmann F, Judex M, Justen HP, Wessinghage D, Gay S, et al. Identification of differentially expressed genes in rheumatoid arthritis by a combination of complementary DNA array and RNA arbitrarily primed polymerase chain reaction. Arthritis Rheum 2002;46:52–63.
26. Wang J, Hu L, Hamilton SR, Coombes KR, Zhang W. RNA amplification strategies for cDNA microarray experiments. Biotechniques 2003;34:394–400.
27. Kamme F, Zhu J, Luo L, Yu J, Tran DT, Meurers B, et al. Single-cell laser-capture microdissection and RNA amplification. Methods Mol Med 2004;99:215–23.
28. Ji W, Zhou W, Gregg K, Lindpaintner K, Davis S, Davis S. A method for gene expression analysis by oligonucleotide arrays from minute biological materials. Anal Biochem 2004;331: 329–39.
29. Judex M, Neumann E, Gay S, Müller-Ladner U. Laser-mediated microdissection as a tool for molecular analysis in arthritis. Methods Mol Med 2004;101:93–105.
30. Tsubaki T, Arita N, Kawakami T, Shiratsuchi T, Yamamoto H, Takubo N, et al. Characterization of histopathology and gene-expression profiles of synovitis in early rheumatoid arthritis using targeted biopsy specimens. Arthritis Res Ther 2005;7:R825–36.
31. Tsubaki T, Takegawa S, Hanamoto H, Arita N, Kamogawa J, Yamamoto H, et al. Accumulation of plasma cells expressing CXCR3 in the synovial sublining regions of early rheumatoid arthritis in association with production of Mig/CXCL9 by synovial fibroblasts. Clin Exp Immunol 2005;141:363–71.
32. Clavel G, Bessis N, Boissier MC. Recent data on the role for angiogenesis in rheumatoid arthritis. Joint Bone Spine 2003;70: 321–6.

Potential New Therapeutic Options for Involvement of Central Nervous System in Behçet's Disease (Neuro-Behçet's Syndrome)

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Abstract: Neuro-Behçet's syndrome consists of acute type and chronic progressive type (primary progressive and secondary progressive). Attacks of acute type neuro-Behçet's syndrome are sometimes self-limiting. However, when the neurological manifestations are progressive and severe, administration of corticosteroid is necessary. In addition, infliximab and interferon alpha might also be effective in acute type neuro-Behçet's disease. There are no drugs which have been demonstrated to be effective in preventing the occurrence of attacks of acute type neuro-Behçet's disease. Colchicine, low dose of steroids and various immunosuppressive drugs have been used anecdotally for this purpose. As to chronic progressive neuro-Behçet's syndrome, one should realize that corticosteroids are not effective. Cyclophosphamide is not effective, either. Low dose methotrexate (MTX) has been shown to be beneficial for the treatment of chronic progressive neuro-Behçet's syndrome by an open clinical trial. Thus, low dose MTX has been shown to decrease cerebrospinal fluid IL-6 levels without progression of neuropsychological manifestations, although there are a fraction of patients who do not adequately respond to MTX. Preliminary results indicate that infliximab has a beneficial effect in such patients with MTX-resistant chronic progressive neuro-Behçet's syndrome.

Keywords: IFN-α, colchicine, cyclosporin A, methotrexate, MRI, infliximab, cerebrospinal fluid, IL-6.

1. INTRODUCTION

Behçet's disease is a chronic relapsing inflammatory diseases of unknown etiology, presenting recurrent aphthous stomatitis, uveitis, genital ulcers, and skin lesions. The predominant histopathological features in the inflamed tissues are infiltration of lymphocytes and monocytes, and sometimes polymorph nuclear leukocytes, through small veins without microscopic changes in the vessel walls. Thrombophilia or thrombophlebilis involving small and large veins is also common, whereas arteritis is rare. In these regards, Behçet's disease is unique compared with other vasculitides [1].

Behçet's disease is characterized by the recurrent episodes of remission and exacerbation of various symptoms, whereas chronic sustained inflammation in certain tissues is rare [1]. Recurrent uveitis attacks usually result in the loss of vision that affects profoundly the activity of daily life of the patients. The involvement of the vascular system, of the intestinal system, and of the central nervous system (CNS) is usually life-threatening, and requires aggressive therapy.

The CNS involvement in Behçet's disease is either caused by primary neural parenchymal lesions (neuro-Behçet's syndrome) or is secondary to major vascular involvement [2, 3]. The latter type is rarely complicated with the parenchymal lesions and should be called vasculo-Behçet's disease [2]. This vasculo-Behçet's disease type generally has a better prognosis compared with the parenchymal type [2].

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The most commonly involved area in neuro-Behçet's syndrome is the brain stem, but spinal cord lesions, cerebral hemisphere lesions and meningoencephalitis also occur frequently [3, 4]. Among a variety of signs and symptoms, pyramidal tract signs are most common [2, 3]. The etiology and pathogenesis of neuro-Behçet's syndrome still remain unclear. In addition, factors determining prognosis and appropriate treatment have not been delineated. However, recent investigations have made significant progress in these areas. Moreover, increasing attention has been paid to the effect of anti-tumor necrosis factor alpha (TNF-α) therapy in this disease [1]. The present article overviews an update on the neuro-pathogenesis, clinical manifestation, and treatment of neuro-Behçet's syndrome, suggesting potential new therapeutic options.

2. CLINICAL MANIFESTATIONS

Headache is the most common neurological symptom seen in Behçet's disease [4, 5]. Although headache is seen in venous sinus thrombosis and uveal inflammation [5], it is also accompanied in the majority of neuro-Behçet's syndrome [5]. Previous studies revealed that the most frequent CNS finding in neuro-Behçet's syndrome was a preference for involvement of brainstem-diencephalon and pontobulbar region [2]. Consistently, motor symptoms, cerebellar symptoms, brainstem symptoms and dysarthria are frequently seen (Table 1) [4]. It should be noted that behavioral symptoms are seen in approximately 10%.

The differential diagnosis of neuro-Behçet's syndrome include many CNS diseases, among which multiple sclerosis was one of the leading misdiagnoses. MRI findings indicate that the major lesion is located in the brainstem-diencephalon-basal ganglion region in neuro-Behçet's syndrome [6]. However, the predominant lesion may be in the periventricular white matter in some cases, where it will be

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difficult to discriminate from multiple sclerosis. In such cases, cerebrospinal fluid (CSF) pleocytosis with polymorphonuclear predominance [6], the absence of more than two oligoclonal IgG bands [6], and the elevation of CSF IL-6 [1] may indicate neuro-Behçet's syndrome. Within patients of Behçet's disease, differential diagnosis should include isolate headache syndromes, cardiogenic embolic stroke, astrocytoma, meningioma, and syringomyelia [4]. Although headache is the most common symptoms in neuro-Behçet's syndrome [4, 5], it is also a common symptom in Behçet's disease independent from neurologic involvement [5]. In addition to careful clinical evaluation, the diagnostic values of CSF and magnetic resonance imaging (MRI) should be prospectively studied.

Table 1. Neurological Symptoms in 164 Patients with Behçet's Syndrome

Headache	101	(61.6%)
Motor symptoms	88	(53.7%)
Cerebellar symptoms other than dysarthria	49	(29.9%)
Brainstem symptoms other than dysarthria	48	(29.3%)
Dysarthria	37	(22.6%)
Behavioral symptoms	20	(12.2%)
Sensory symptoms	18	(11%)
Alteration of consciousness	12	(7.3%)
Cognitive symptoms	4	(2.4%)
Others (seizures, peripheral neuropathy, optic neuritis, etc)	16	(9.8%)

The 164 patients include 20 patients with venous sinus thrombosis 124 patients with neuro-Behcet's syndrome. (modified from ref. [4]).

We have recently disclosed that neuro-Behçet's syndrome can be classified into acute type and chronic progressive type [7]. Acute neuro-Behçet's syndrome is characterized by acute meningoencephalitis with or without focal lesions, presenting high-intensity areas in T2-weighted images or fluid attenuated inversion recovery (FLAIR) images on MRI scans [7] (Fig. 1). Cyclosporin A is frequency associated with acute type neuro-Behçet's syndrome, at least among the Japanese patients [8]. Acute type neuro-Behçet's syndrome responds to corticosteroid therapy, and is usually self-limiting, although recurrence of the attacks is sometimes seen. It should be noted, however, that there are still patients with high degree of permanent damage or disability due to acute type attacks [4, 6, 9].

By contrast, the chronic progressive type of neuro-Behçet's syndrome is characterized by intractable, slowly progressive neurobehavior changes, ataxia and dysarthria, leading to severe disability and deteriolation (Table 2) [1, 4]. The neurobehavior changes include cognitive dysfunction, euphoria, loss of insight, disinhibition, indifference to their disease, psychomotor agitation or retardation, with paranoid attitudes and obsessive concerns [4]. These symptoms should not be confused with psychosis associated with the use of corticosteroid or other therapy. Of note, the patients with the chronic progressive type of neuro-Behçet's syndrome show persistent marked elevation of CSF IL-6 (>20 pg/ml) with

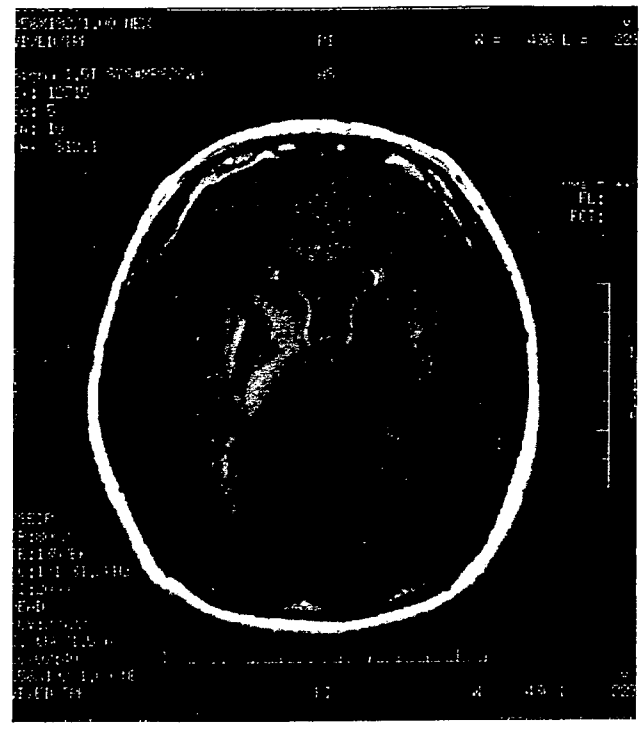

Fig. (1). Axial fluid attenuated inversion recovery (FLAIR) brain MRI of a patient with acute type neuro-Behçet's syndrome, showing high density lesions in the right internal and external capsules.

Table 2. Clinical Features of Chronic Progressive Type Neuro-Behçet's Syndrome: Summary of 11 Cases

Neurological manifestations	
Dementia/psychosis	11 cases
Ataxia	10 cases
Dysarthria	9 cases
Urinary incontinence	7 cases
Myoclonus	1 cases
MRI findings	
Atrophy	
Brainstem/cerebellum	10 cases
Cerebrum	4 cases
Scattered T2 high lesions	5 cases

very modest increase in cell numbers and total protein (Fig. 2) [10]. No significant elevation of serum IL-6 was noted in patients with chronic progressive neuro-Behçet's syndrome. The MRI findings may show only atrophy of brain stem and cerebellum in the chronic progressive neuro-Behçet's syndrome (Fig. 3). Most patients (approximately 90%) in our series with the chronic progressive type of neuro-Behçet's syndrome were HLA-B51-positive, and they had history of attacks of acute type neuro-Behçet's syndrome prior to the development of progressive neuropsychological symptoms [10]. Recently, it has been disclosed that HLA-B51 and cigarette smoking, and especially their combination, are risk fac-

tors for chronic progressive neuro-Behçet's syndrome [11]. It is likely that certain substances in cigarettes might be immunogenic in the context of HLA-B51, resulting in the persistent activation of immune responses within the CNS in Behçet's disease [11].

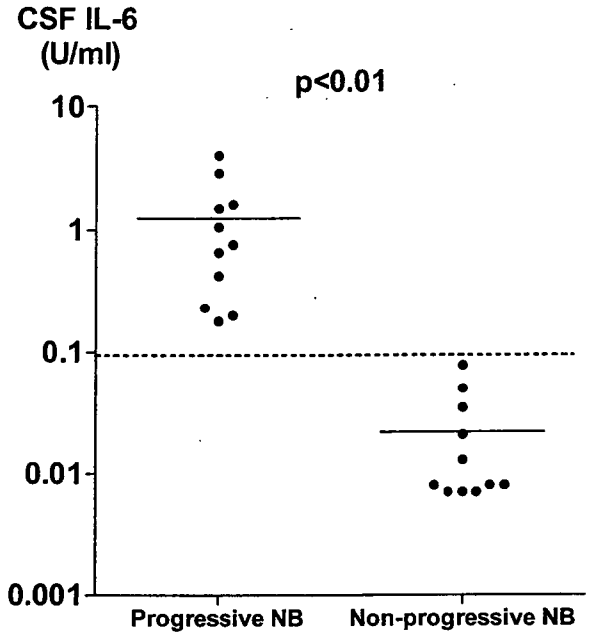

Fig. (2). Marked elevation of cerebrospinal fluid (CSF) IL-6 in patients with chronic progressive type neuro-Behçet's syndrome (NB). CSF samples were analyzed for IL-6 activity using IL-6 dependent murine hybridoma MH60.BSF2 cells. CSF IL-6 0.1 U/ml is equivalent to 20 pg/ml (Modified from ref. [10]).

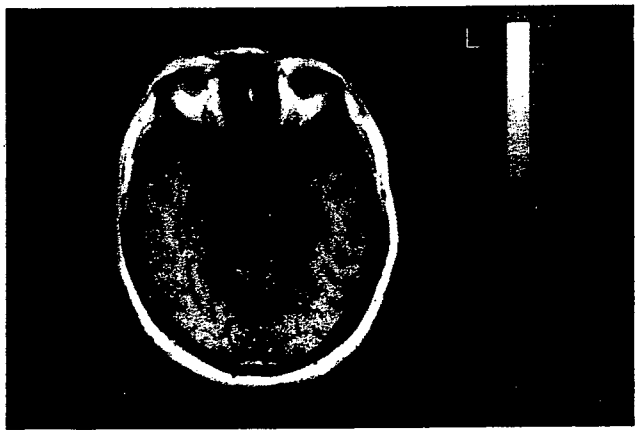

Fig. (3). Axial fluid attenuated inversion recovery (FLAIR) brain MRI of a patient with chronic progressive type neuro-Behçet's syndrome, showing atrophy of brainstem and cerebellum.

These two types of neuro-Behçet's syndrome are currently considered to represent different stages rather than be independent clinical entities. In fact, we have recently experienced some patients who displayed prolonged elevation of CSF IL-6 activity following the acute type neuro-Behçet's syndrome. It is therefore suggested that the appropriate treatment of such patients can prevent progression of neuropsychological symptoms, although further studies are required to confirm this point. Of note, chronic progressive neuro-Behçet's syndrome is resistant to conventional treatment with corticosteroid, with cyclophosphamide, or with azathioprine [10, 12]. Recent studies, however, suggest the efficacy of low-dose weekly methotrexate (MTX) in the chronic progressive type of neuro-Behçet's syndrome [12], as will be discussed later.

Akman-Demir *et al.* proposed the subsets of neuro-Behçet's syndrome, including attack(s) and remission, secondary progression, primary progression, and silent neurological involvement [6]. Attack(s) and remission in their series are considered to correspond to acute type, whereas primary and secondary progression should be the some as chronic progressive type in our classification [7]. Thus, most of our patients with chronic progressive neuro-Behçet's syndrome had preceeding history of acute attacks [7]. It is also likely that some patients with silent neurological involvement might represent preceding symptoms of primary progressive courses [6]. Of note, some patients with silent neurological involvement later developed an attack in the series of Akman-Demir and colleagues [6]. It is therefore likely that silent neurological involvement might be a modest form of acute type neuro-Behçet's syndrome in our classification [7].

3. TREATMENT

Treatment of neuro-Behçet's syndrome may be different depending on its presentation: acute type and chronic progressive type. As to acute type neuro-Behçet's syndrome, treatment of active phase manifestations as well as prevention of another attack should be considered separately [4].

a. Acute Type: Active Phase Manifestations

Corticosteroids are used to treat active phase in acute type neuro-Behçet's syndrome, but their effects are short-lived and they do not prevent further attacks or progression. The patients are usually treated with oral prednisolone (1 mg/kg for up to 4 weeks, or until improvement is observed) with or without high-dose intravenous methylprednisolone (1 g/day) for 3-7 days [13]. Both forms of treatment should be followed with an oral tapering dose of corticosteroids over 2-3 months in order to prevent early relapses [13]. Although high dose intravenous methylprednisolone has been suggested to result in earlier improvement, there has been no controlled study to demonstrate its efficacy.

In addition to corticosteroid, the efficacy of interferon alpha (IFN-α) has been implicated in the treatment of the active phase manifestations of acute type neuro-Behçet's disease [14]. Thus, Nichols *et al.* treated a 25-year-old male patient with a generalized tonic-clonic seizure who showed a right temporal lobe lesion on MRI scan with IFN-α2a (9 million units administered subcutaneously three times per week for 5 weeks; total dose approximaterly 135 million units) along with colchicine (1.2 mg/day). Neurologic manifestations markedly improved as the temporal lobe lesion improved (evidenced by MRI scan), and disc edema nearly resolved at the 6-week follow-up. Repeated MRI scan of the brain and ocular examination at approximately 3 months showed resolution of the temporal lobe lesion, disc edema, intraocular inflammation and systemic, ocular, and neurologic manifestations through the 8-month follow-up period, maintained only on colchicine (1.2 mg/day) [14]. Thus, IFN-α2a might be effective in the treatment of acute type

neuro-Behçet's syndrome as well as ocular manifestations, although further controlled study is required to confirm this point.

Recently, infliximab, a chimeric monoclonal antibody to TNF-α, has been demonstrated to be effective for Crohn's disease [15] and rheumatoid arthritis [16]. Accumulating reports on patients with Behçet's disease showed that infliximab was effective in the treatment of intractable orogenital ulceration [17], of skin lesions [18], and of gastrointestinal lesions [19]. It has also been disclosed that infliximab is a rapid and effective therapy for sight-threatening panuveitis in Behçet's disease [20]. In fact, recent clinical trials have demonstrated that infliximab is effective in suppressing the frequency of ocular attacks, and has favorable implications for the visual prognosis of patients with refractory uveoretinitis [21, 22]. Recent reports have also suggested the efficacy of infliximab for recalcitrant acute type neuro-Behçet's disease [23, 24]. Thus, Licata *et al.* treated a 59-year-old female patient, who developed multiple large cortical-subcortical lesions in spite of intravenous methylprednisolone and cyclophosphamide therapy, with infliximab (5 mg/kg at weeks 0, 2, and 6) without any adverse effects. An improvement in symptoms was noticed within 24 hours after receiving the first infusion and remained stable throughout the observation period. Cerebral MRI scans performed a week after the infusion showed a complete resolution of signal abnormalities. After the second infusion the patient had a complete remission of all signs and symptoms of neurological involvement [23]. A similar patient with brainstem lesions in acute neuro-Behçet's syndrome has been reported to respond well to infliximab (3mg/kg at weeks 0, 2, and 6) [24].

On the other hand, it should be pointed out that the neurological involvement of acute type neuro-Behçet's disease sometimes subsides spontaneously. Thus, a 37-year-old Japanese male patient, who developed meningoencephalitis with consciousness disturbances, improved spontaneously without administration of corticosteroid (Fig. 4) [25]. Another 55-year-old Japanese female patient with fever and headache showed high intensity lesions on MRI scans (Fig. **5a**). She improved spontaneously in a month with conventional treatment with colchicine alone (Fig. **5b**). However, she showed the evidence of minor attack on MRI scans in spite of the lack of any symptoms 6 months later (data not shown). Taken together, the use of corticosteroid, IFN-α and infliximab should be considered depending on the severity and the course of the neurological manifestations of patients with acute type neuro-Behçet's disease, since spontaneous remission might occur in some patients. Thus, careful evaluation of the severity of neurological attacks would be important.

b. Acute Type: Prevention of Relapse

Colchicine, azathioprine, cyclosporin A, cyclophosphamide, methotrexate, chlorambucil, and immunomodulatory agents such as IFN-α, pentoxyphilline and thalidomide have been anecdotally shown to be beneficial in preventing the occurrence of the systemic manifestations of Behçet's disease, but none of these agents have been shown to be effective in preventing the attacks of acute type neuro-Behçet's

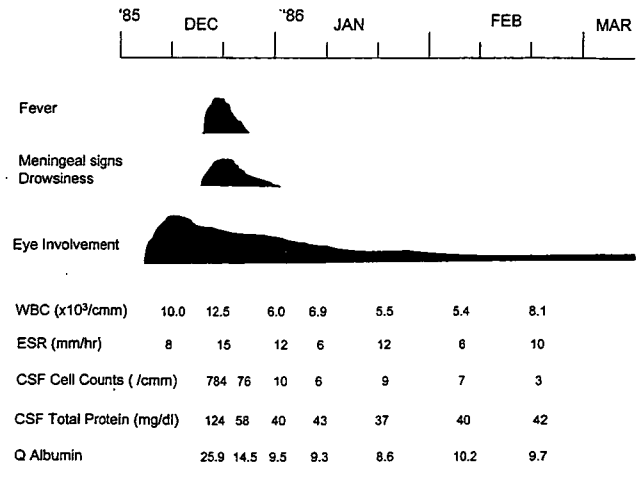

Fig. (4). Clinical course and changes in laboratory data of a patient with acute type neuro-Behçet's syndrome, who showed spontaneous remission (Modified from ref. [25]).

syndrome in a properly designed study [4]. Cyclosporin was reported to cause neurotoxicity or to accelerate the development of CNS symptoms, and therefore its use in neuro-Behçet's syndrome is not recommended [4, 8].

Shown in Table **3** is our experience of follow up of 10 patients with acute type neuro-Behçet's syndrome. Four of the 10 patients were taking cyclosporin A when they developed acute attack, and most patients showed abnormal densities on MRI scans. Almost all the patients were followed up with tapering doses of prednisolone and colchicine. Although minor relapsing attacks were noted in 2 of the 10 patients, most patients were dosing well during the follow-up periods. Although one patient continued to take cyclosporin A due to recurrent uveitis, it is not generally recommended because of its neurotoxicity [4, 8].

c. Chronic Progressive Type

Addition of immunosuppressive drugs, such as azathioprine, oral or intravenous cyclophosphamide to high doses corticosteroid has been usually performed in progressive neuro-Behçet's syndrome cases; however, the efficacy of such a combination has also not been demonstrated to date [4, 6]. In most patients with chronic progressive neuro-Behçet's syndrome, high doses of corticosteroid are only partially effective. Thus, the patients usually show exacerbation along with the elevation of CSF IL-6 activity when the doses of corticosteroid are decreased. We have also found that cyclophosphamide, given orally or intravenously, has no effect. A 37-year-old Japanese male patient with chronic progressive neuro-Behçet's disease, who were treated with high doses of corticosteroid, repeated methylprednisolone pulse therapy and repeated intravenous cyclophosphamide, did not recover from the neurological manifestations, and eventually died of infection due to steroid-induced immunodeficiency. It should be noted that CSF IL-6 activity never dropped below 0.1 U/ml (20 pg/ml) throughout the course (Fig. 6). The course of this patient therefore confirms the lack of efficacy of high doses of corticosteroid and intravenous cyclophosphamide in chronic progressive type neuro-Behçet's syndrome.

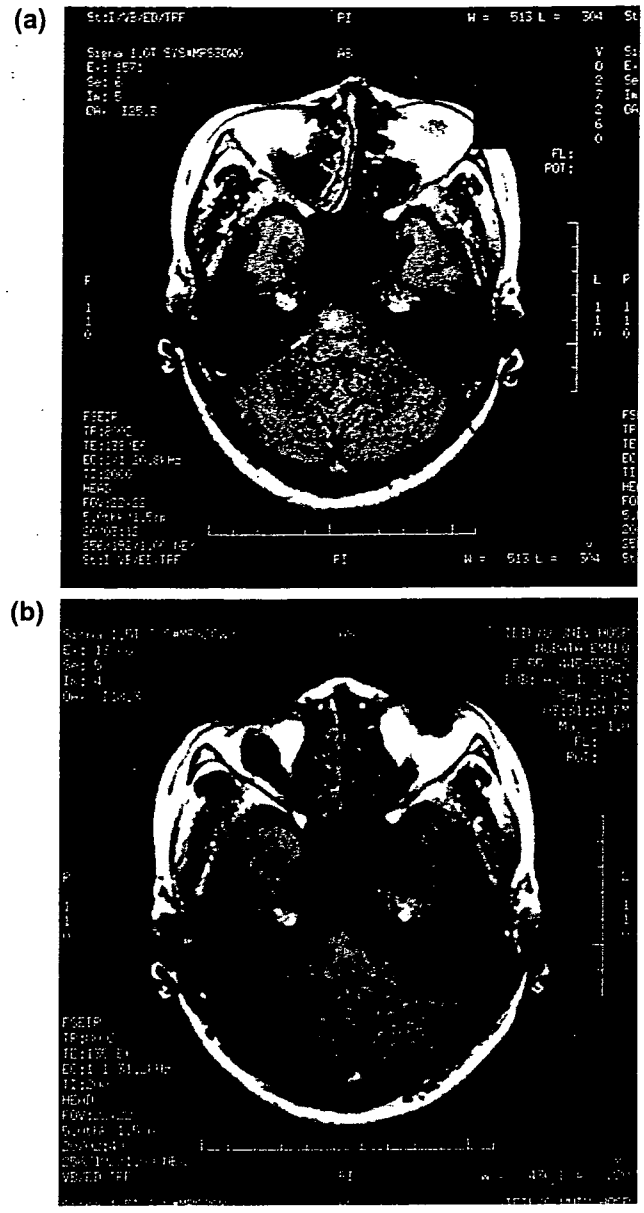

Fig. (5). Axial fluid attenuated inversion recovery (FLAIR) brain MRI of a patient with acute type neuro-Behçet's syndrome at acute phase (**a**), and remission phase (**b**).

We happened to experience a patient with chronic progressive type neuro-Behçet's syndrome, in whom low dose weekly MTX dramatically decreased the CSF IL-6 activity. An open trial was therefore designed to investigate the efficacy of low dose weekly MTX therapy for progressive NB [12]. Six patients with Behçet's disease, whose neuropsychiatric manifestations were judged to be progressive (4 females and 2 males, aged 55.0 ± 8.2 years), were given oral MTX (7.5-12.5 mg/week) until the end of the 12-month trial. After the 12-month trial, CSF IL-6 levels were found to be significantly decreased. Accordingly, the neuropsychological manifestations as well as the findings on MRI scans and intelligence quotients were not significantly worsened after the trial [12]. Three patients presented with mild liver dysfunction, which returned to normal by decreasing the dose of MTX. However, 6 months after discontinuation of MTX, all the six patients showed significant exacerbation of the manifestations as evidenced by a decrease in verbal intelligence quotients along with the marked elevation of CSF IL-6 [12]. These results suggest that low dose weekly MTX therapy might have a beneficial effect in the treatment of chronic progressive type neuro-Behçet's syndrome.

Further studies were performed to investigate the efficacy and the safety of low-dose weekly MTX for a longer period (4 years). Ten patients with chronic progressive neuro-Behçet's syndrome were given oral MTX until the end of the 4-year-trial. The initial dose of MTX was 5.0-7.5 mg/week, and the dose was increased by 2.5 mg every 2 weeks up to 5.0-15.0 mg/week according to CSF IL-6 concentrations (the dose was adjusted so that CSF IL-6 was decreased below 20 pg/ml). The patients were allowed to continue to take small doses of prednisolone (less than 15 mg/day) and colchicine (1.0 mg/day) without changes of doses. Two of the 10 patients dropped out of the trial due to complications other than MTX toxicity. During 4 years of the trial, CSF IL-6 levels were significantly decreased (Fig. 7). In addition, the neuropsychiatric manifestations as well as the findings on MRI scans and intelligence quotients were not significantly worsened (Fig. 8). Three patients presented mild liver dysfunction, which returned to normal by decreasing the doses of MTX or supplementing folate (5.0-10 mg/week) [26]. The results suggest that low dose weekly MTX therapy might be tolerable and have a beneficial effect in the treatment of patients with chronic progressive type neuro-Behçet's syndrome, since it prevented the progression of the neuropsychiatric manifestations by significantly decreasing CSF IL-6 levels. It should be emphasized, however, there are still a fraction of patients with chromic progressive neuro-Behçet's syndrome, who did not adequately respond to MTX [26].

Infliximab has been shown to be effective for the treatment of acute type neuro-Behçet's disease. Of note, it has also been reported that infliximab was effective for the treatment of long-standing neuro-Behçet's disease, chronic progressive type. Thus, Sarwar *et al.* reported a 42-year-old man who showed prolonged episodes of slurred speech with lack of orientation to time, place, and person. The patient improved markedly after administration of infliximab (3 mg/kg) with prednisolone 60 mg/day. The patient received infusion of infliximab for three times, and the dose of prednisolone was successfully tapered to 10 mg/day without exacerbation after one year follow up [27]. Recently, we have also treated a 29-year-old Japanese male with chronic progressive neuro-Behçet's syndrome, who showed persistent elevation of IL-6 despite of treatment with prednisolone 10 mg/day and methotrexate 17.5 mg/week, with infliximab (5 mg/kg). Surprisingly, the CSF IL-6 level was markedly decreased from 4.884 U/ml to 0.280 U/ml on the next day of the infusion of infliximab. It is therefore suggested that TNF-α might play a critical role in the pathogenesis of neuro-Behçet's disease at the upstream of IL-6 [Kikuchi H, Takayama M, Arinuma Y, Aramaki K, Komagata Y, Hirohata S: Infliximab for progressive neuro-Behcet's syndrome refractory to methotrexate. 12th International Conference on Behcet's disease. Lisbon, S-33, 2006.]. Etanercept, a recombinant TNF receptor Fc fusion protein, is also now being used in Behçet's disease. Thus, recent studies have demonstrated that etanercept was effective in suppressing most of the mucocutaneous manifestations in Behçet's disease [28]. A clinical trial to confirm the efficacy of infliximab and

Table 3. Follow-Up of 10 Patients with Acute Type Neuro-Behçet's Syndrome

Patients	Acute Attack	CyA at Onset	MRI Abnormal Densities	Acute-Phase Treatment	Follow-Up Treatment	Years	Outcome
38 M	lt hemiparesis, dysarthria	(+)	(+)	PSL 30 mg/day	Colchicine, tapering PSL (0)	9	No relapse
59 F	rt. hemiparesis, headache, fever	(-)	(+)	PSL 30 mg/day	Colchicine, tapering PSL (5)	8	No relapse
17 M	headache, fever	(+)	(+)	PSL 60 mg/day	Colchicine, tapering PSL (10)	8	No relapse
59 M	ataxia, dysarthria	(+)	(+)	mPSL pulse + PSL 60 mg/day	Colchicine, tapering PSL (1.5)	9	No relapse
40 M	headache, fever	(-)	(+)	mPSL pulse + PSL 30 mg/day	Colchicine, tapering PSL (2.5)	5	No relapse
29 F	nausea, vertigo, vomiting	(-)	ND	PSL 40 mg/day	Colchicine, tapering PSL (0)	11	One minor attack at one year
29 F	headache, fever, nausea	(-)	ND	PSL 20 mg/day	Colchicine, tapering PSL (0)	6	No relapse
47 M	headache, fever	(+)	(+)	PSL 30 mg/day	Colchicine, tapering PSL (0), CyA	13	No relapse
34 F	seizure	(-)	(+)	mPSL pulse + PSL 60 mg/day	tapering PSL (0)	4	No relapse
55 F	headache, fever	(-)	(+)	not in particular	Colchicine, Intermittent PSL (0-5)	3	One minor relapse without symptoms

CyA = cyclosporin A, ND = not done, PSL = prednisolone, mPSL = methylprednisolone.

etanercept in the treatment of chronic progressive neuro-Behçet's syndrome is therefore warranted.

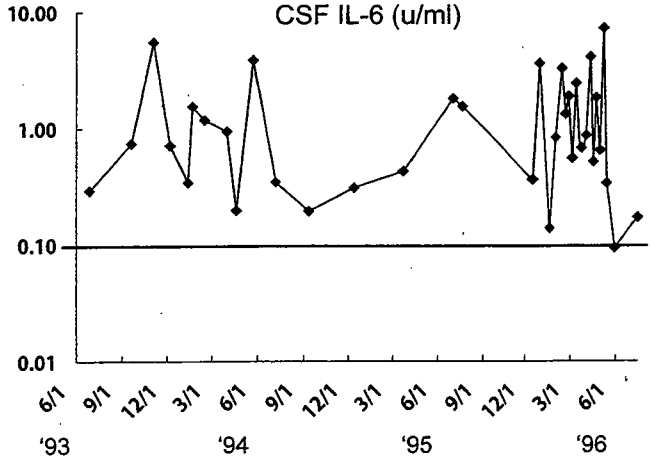

Fig. (6). Changes of CSF IL-6 levels in a 37-year-old Japanese male patient who died of chronic progressive type neuro-Behçet's syndrome. Note that CSF IL-6 has never been decreased below 0.1 U/ml (20 pg/ml) throughout the course.

4. CONCLUSION

Neuro-Behçet's syndrome consists of acute type and chronic progressive type. In acute type, administration of corticosteroid should be considered depending on the severity of the attack. In addition, infliximab and IFN-α might also be effective for subsiding the acute attack. Colchicine, low doses of steroids and various immunosuppressive drugs have been used anecdotally for prevention of the recurrence of attacks of acute type neuro-Behçet's disease. As to chronic progressive neuro-Behçet's syndrome, one should realize that corticosteroids and cyclophosphamide are not effective. Low dose MTX has been shown to be effective for chronic progressive neuro-Behçet's syndrome. In addition, infliximab appears to be promising.

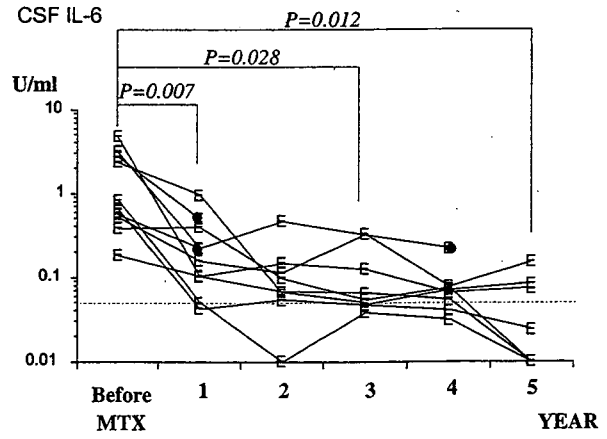

Fig. (7). Cerebrospinal fluid (CSF) IL-6 in patients with chronic progressive type neuro-Behçet's syndrome. Solid circle indicates the points of drop out. Statistical analysis was done by Wilcoxon signed rank test (from ref. [26]).

ABBREVIATIONS

CNS = Central nervous system

CSF = Cerebrospinal fluid

FLAIR = Fluid attenuated invention recovery

IFN-α = Interferon alpha

MTX = Magnetic resonance imaging

MTX = Methotrexate

TNF-α = Tumor necrosis factor alpha

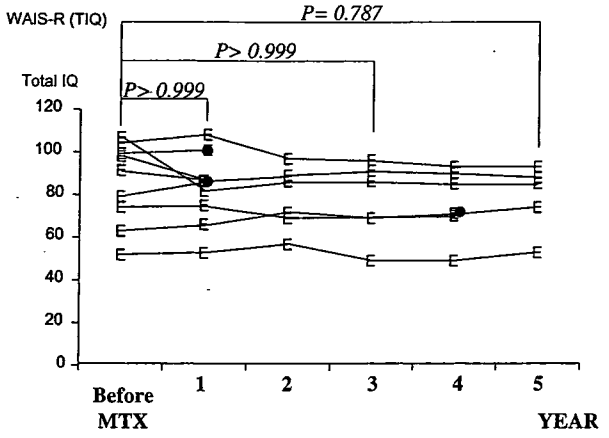

Fig. (8). Results of revised Wechsler adult intelligence scale in patients with chronic progressive type neuro-Behçet's syndrome. TIQ: total intelligence quotient. Solid circle indicates the points of drop out. Statistical analysis was done by Wilcoxon signed rank test (From ref. [26]).

REFERENCES

[1] Hirohata S, Kikuchi H. Behçet's disease. Arthritis Res Ther 2003; 5: 139-46.

[2] Serdaroglu P. Behçet's disease and the nervous system. J Neurol 1998; 245: 197-205.

[3] Kidd D, Steuer A, Denman AM, Rudge P. Neurological complications in Behçet's syndrome. Brain 1999; 122: 2183-94.

[4] Siva A, Altintas A, Saip S. Behçet's syndrome and the nervous system. Curr Opin Neurol 2004; 17: 347-57.

[5] Saip S, Siva A, Altintas A, *et al.* Headache in Behçet's syndrome. Headache 2005; 45: 911-9.

[6] Akman-Demir G, Serdaroglu P, Tasci B. The Neuro-Behçet Study Group. Clinical patterns of neurological involvement in Behçet's disease: evaluation of 200 patients. Brain 1999; 122: 2171-82.

[7] Kawai M, Hirohata S. Cerebrospinal fluid beta(2)-microglobulin in neuro-Behcet's syndrome. J Neurol Sci 2000; 179: 132-9.

[8] Kotake S, Higashi K, Yoshikawa K, Sasamoto Y, Okamoto T, Matsuda H. Central nervous system symptoms in patients with Behçet disease receiving cyclosporine therapy. Ophthalmology 1999; 106: 586-9.

[9] Lo Monaco A, La Corte R, Caniatti L, Borrelli M, Trotta F. Neurological involvement in North Italian patients with Behçet disease. Rheumatol Int 2006; 26: 1113-9.

[10] Hirohata S, Isshi K, Oguchi H, *et al.* Cerebrospinal fluid interleukin-6 in progressive Neuro-Behcet's syndrome. Clin Immunol Immunopathol 1997; 82: 12-7.

[11] Aramaki K, Kikuchi H, Hirohata S. HLA-B51 and cigarette smoking as risk factors for chronic progressive neurological manifestations in Behcet's disease. Mod Rheumatol 17:81-2, 2007.

[12] Hirohata S, Suda H, Hashimoto T. Low-dose weekly methotrexate for progressive neuropsychiatric manifestations in Behçet's disease. J Neurol Sci 1998; 159: 181-5.

[13] Siva A, Fresko I. Behçet's Disease. Curr Treat Options Neurol 2000; 2: 435-48.

[14] Nichols JC, Ince A, Akduman L, Mann ES. Interferon-alpha 2a treatment of neuro-Behçet disease. J Neuroophthalmol 2001; 21: 109-11.

[15] Targan SR, Hanauer SB, van Deventer SJ, *et al.* A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. N Engl J Med 1997; 337: 1029-35.

[16] Maini R, St Clair EW, Breedveld F, *et al.* Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT Study Group. Lancet 1999; 354: 1932-9.

[17] Robertson LP, Hickling P. Treatment of recalcitrant orogenital ulceration of Behçet's syndrome with infliximab. Rheumatology (Oxford) 2001; 40: 473-4.

[18] Goossens PH, Verburg RJ, Breedveld FC. Remission of Behçet's syndrome with tumour necrosis factor alpha blocking therapy. Ann Rheum Dis 2001; 60: 637.

[19] Hassard PV, Binder SW, Nelson V, Vasiliauskas EA. Anti-tumor necrosis factor monoclonal antibody therapy for gastrointestinal Behçet's disease: a case report. Gastroenterology 2001; 120: 995-9.

[20] Sfikakis PP, Theodossiadis PG, Katsiari CG, Kaklamanis P, Markomichelakis NN. Effect of infliximab on sight-threatening panuveitis in Behçet's disease. Lancet. 2001; 358: 295-6.

[21] Ohno S, Nakamura S, Hori S, *et al.* Efficacy, safety, and pharmacokinetics of multiple administration of infliximab in Behçet's disease with refractory uveoretinitis. J Rheumatol 2004; 31: 1362-8.

[22] Tugal-Tutkun I, Mudun A, Urgancioglu M, *et al.* Efficacy of infliximab in the treatment of uveitis that is resistant to treatment with the combination of azathioprine, cyclosporine, and corticosteroids in Behçet's disease: an open-label trial. Arthritis Rheum 2005; 52: 2478-84.

[23] Licata G, Pinto A, Tuttolomondo A, *et al.* Anti-tumour necrosis factor alpha monoclonal antibody therapy for recalcitrant cerebral vasculitis in a patient with Behçet's syndrome. Ann Rheum Dis 2003; 62: 280-1

[24] Fujikawa K, Aratake K, Kawakami A, *et al.* Successful treatment of refractory neuro-Behcet's disease with infliximab: a case report to show its efficacy by magnetic resonance imaging, transcranial magnetic stimulation and cytokine profile. Ann Rheum Dis. 2007; 66: 136-7..

[25] Hirohata S, Kamoshita H, Taketani T. Spontaneous remission of meningoencephalitis in Behçet's disease. J Rheumatol 1989; 16: 1283-4.

[26] Kikuchi H, Aramaki K, Hirohata S. Low dose MTX for progressive neuro- Behçet's disease. A follow-up study for 4 years. Adv Exp Med Biol 2003; 528: 575-8.

[27] Sarwar H, McGrath H Jr, Espinoza LR. Successful treatment of long-standing neuro-Behcet's disease with infliximab. J Rheumatol 2005; 32: 181-3.

[28] Melikoglu M, Fresko I, Mat C, *et al.* Short-term trial of etanercept in Behçet's disease: a double blind, placebo controlled study. J Rheumatol 2005; 32: 98-105.

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関節リウマチの治療

そのほかの抗リウマチ薬の使い方と副作用
(金製剤，D-ペニシラミン，ブシラミン，サラゾスルファピリジン)

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SUMMARY

メトトレキサート以外の抗リウマチ薬について，その使用法と副作用を中心に概説した．注射金剤（シオゾール®）のみ筋注で用いられる．副作用としては，経口金剤〔オーラノフィン（リドーラ®）〕では胃腸障害の頻度が高い．重篤な副作用としては，シオゾール・D-ペニシラミン（メタルカプターゼ®）・ブシラミン（リマチル®）ではとくに腎障害（膜性腎症）に注意する必要がある．サラゾスルファピリジン（アザルフィジン®EN）に特有の副作用として光線過敏症が見られる．

はじめに

関節リウマチ（RA）は，関節滑膜の慢性増殖性炎症を経て，関節の骨・軟骨の破壊にいたる疾患である．RAの病態形成において，免疫反応の異常が関与することが明らかとなって以来，従来からの基礎療法を底辺としたピラミッド型のRAの治療体系が見直され，病初期よりその免疫異常を是正するための免疫調節薬の積極的使用が行われるようになってきている．これらの免疫調節薬は，RAにおける病状の進展を変えうるものであるという期待から，DMARD（disease modifying antirheumatic drugs）あるいは単に抗リウマチ薬と総称され，非ステロイド系消炎鎮痛薬（NSAID）と区別されている．最も基本となるDMARDはメトトレキサート（MTX）であり，本薬はRA治療のアンカードラッグとしての位置を確立したといってよい．本稿では，MTX以外のDMARDのなかで，金製剤，D-ペニシラミン，ブシラミン，サラゾスルファピリジンの使い方と副作用について概説する．

Ⅰ．金製剤

① 基礎的事項

19世紀末から20世紀初めにかけて結核の治療薬として用いられていた注射金剤が，1920年代後半よりRAの治療に用いられるようになった[1]．以来70年以上にわたって使用され続けていることは，この薬剤の持つ捨てがたい臨床的有用性を示している．

経口金剤〔オーラノフィン（リドーラ®）〕は注射

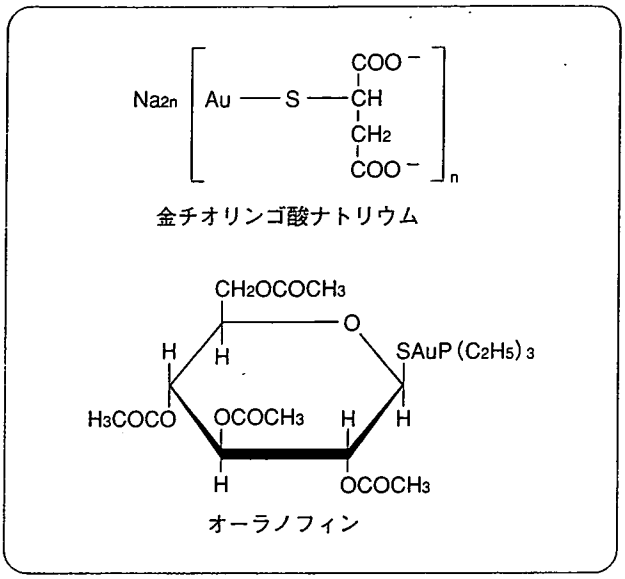

図1　注射金剤（金チオリンゴ酸ナトリウム）と経口金剤（オーラノフィン）の構造式

表1　金製剤の副作用

	注射金剤（シオゾール®）	経口金剤（オーラノフィン）
とくに頻度の高いもの	皮　疹	消化器症状
	口内炎	（下痢・軟便・腹痛）
	蛋白尿	皮　疹
	顕微鏡的血尿	口内炎
	ネフローゼ症候群	**骨髄障害**
	剥脱性皮膚炎	**間質性肺炎**
	再生不良性貧血	**肝障害**
	間質性肺炎	**腎障害**
	血管運動性反応	
	肝障害	

太字は重篤となりうるものを示す．

金剤の注射の繁雑さを解消するために1980年代に開発された[2]．一般に，経口金剤は注射金剤〔金チオリンゴ酸ナトリウム（シオゾール®）〕に比して副作用は少ないが効果も劣っているという評価が定着している．

図1に注射金剤と経口金剤の構造式を示す．同じ金製剤でもその性質はかなり異なっている．ちなみに注射金剤は水に溶けやすいのに対して，経口金剤はまったく水に溶けない．むしろ，注射金剤は分子内にSH基を有する点で，後述するD-ペニシラミンやブシラミンに類似しているともいえる．

② 使い方

シオゾール®の近年における使用法は，低用量投与法が一般的である．通常は，初回10mg/週のペースで毎週筋注し，2～4週間後に25mgに増量し，投与間隔を2～4週に1回とする方法が行われている．オーラノフィンは一回1錠3mgを朝夕2回内服する方法が一般的である．

③ 臨床的効果

シオゾール®は蓄積性があり，その効果発現は遅効性で，通常総量200mg以上に達してから効果が発現し始める．オーラノフィンも遅効性であり，その効果発現までには約2ヵ月を要する．これは，オーラノフィン6mg/日の反復投与を続けたときに血中金濃度が定常状態（0.672μgAu/mL）に達する期間とほぼ一致している．

いったんシオゾール®で寛解導入に成功した例でも，注射を継続しているにもかかわらず開始後2～4年頃より効果が減弱する場合が多い．これは何らかの耐性現象ではないかと考えられているが，その詳細な機序は不明である．オーラノフィンでも同様の耐性現象はしばしば経験されるが，その発現はシオゾール®よりも早期よりみられる傾向にある．

④ 副作用

主な副作用を表1に示す．副作用の出現頻度は，シオゾール®もオーラノフィンも軽いものから重篤なものまで含めて約30～50%である．シオゾール®では，膜性糸球体腎炎による蛋白尿，顕微的血尿などがよく見られるが，オーラノフィンでは少ない．これらは，シオゾール®の中止だけ

では軽快せず，副腎皮質ステロイドの使用を余儀なくされることも少なくない．

シオゾール®，オーラノフィンのいずれにおいても，まれな副作用であるが重篤なものとして，間質性肺炎，骨髄障害が見られるので注意が必要である．

Ⅱ．D-ペニシラミン(メタルカプターゼ®)とブシラミン(リマチル®)

① 基礎的事項

メタルカプターゼ®(DPC)とリマチル®(BUC)はいずれも分子内にSH基を有するいわゆるSH化合物である(図2)[3]．事実，この両薬剤の効果発現までの期間・臨床効果・副作用などについては類似している点も多い．しかし，この両薬剤は構造上決定的な違いを有している．すなわち，DPCは分子内にSH基を1つしかもたないのに対し，BUCは同一分子内にSH基を2つ有しているという点である．このために，BUCを投与された患者においては生体内でいくつかのユニークな代謝産物が形成されることが明らかになっている．そのなかには，2つのSH基が分子内S-S結合を形成したSA981，2つのSH基のうち1つがメチル化されたSA679，SH基が2つともメチル化されたSA672が含まれる[3]．このような代謝経路以外にも，DPCおよびBUCは分子間でS-S結合を形成することが知られている．

BUCの代謝体のSA981はDPCには見られない強力なBリンパ球抑制効果をはじめとするユニークな作用があり，これが両者の臨床効果の決定的な差を生じているといっても過言ではない[4]．

② 使い方

DPCは通常50～100mg/日より開始して漸増し，200～300mg/日程度で維持する(最大600mg/日)．ビタミンB6の代謝を阻害するので，リン酸ピリドキサール(ピドキサール®)を20～30mg/日で併用する．

一方，BUCが一般臨床に使用され始めた当時，

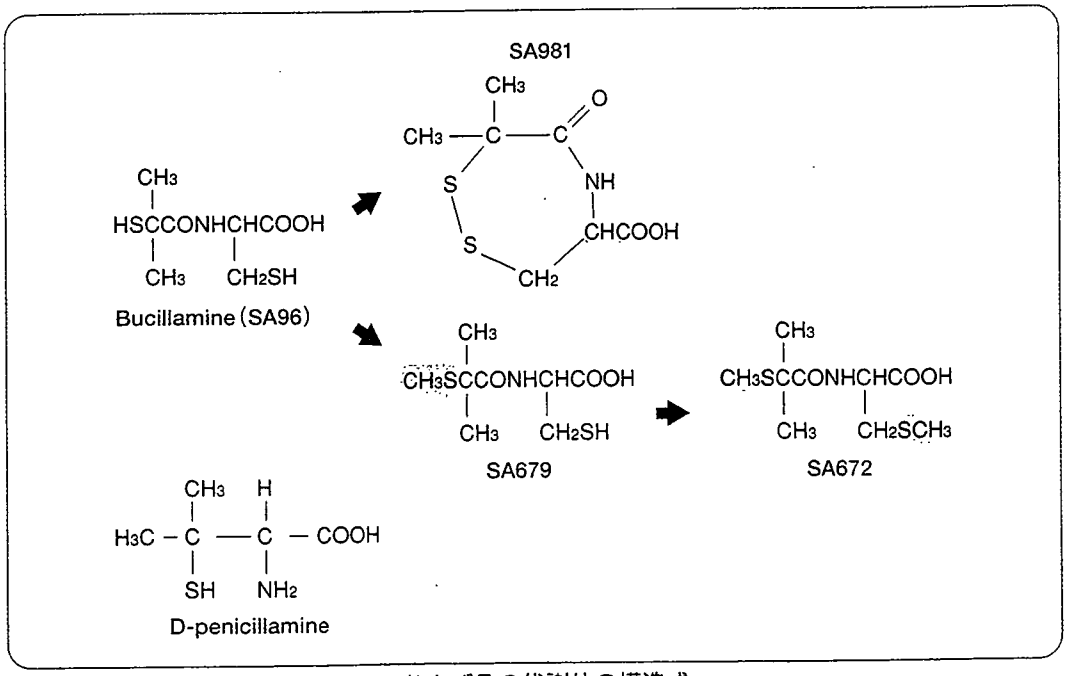

図2　D-ペニシラミン，ブシラミンおよびその代謝体の構造式