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Ⅴ．研究成果の刊行物・別刷

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The regulatory role of natural killer cells in multiple sclerosis

Kazuya Takahashi,[1] Toshimasa Aranami,[1] Masumi Endoh,[1,2] Sachiko Miyake[1] and Takashi Yamamura[1]

[1]*Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502 and* [2]*Department of Bioregulation, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba, Higashimurayama, Tokyo 189-0002, Japan*

Correspondence to: Takashi Yamamura, Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
E-mail: yamamura@ncnp.go.jp

Summary

Multiple sclerosis is a chronic demyelinating disease of presumed autoimmune pathogenesis. The patients with multiple sclerosis typically shows alternating relapse and remission in the early stage of illness. We previously found that in the majority of multiple sclerosis patients in a state of remission, natural killer (NK) cells contain unusually high frequencies of the cells expressing CD95 (Fas) on their surface (>36.0%). Here we report that in such 'CD95$^+$ NK-high' patients, NK cells may actively suppress potentially pathogenic autoimmune T cells that can mediate the inflammatory responses in the CNS. Using peripheral blood mononuclear cells (PBMCs) derived from 'CD95$^+$ NK-high' or 'CD95$^-$ NK-low' multiple sclerosis in a state of remission, we studied the effect of NK cell depletion on the memory T cell response to myelin basic protein (MBP), a major target antigen of multiple sclerosis. When we stimulated PBMCs of the 'CD95$^+$ NK-high' multiple sclerosis after depleting CD56$^+$ NK cells, a significant proportion of CD4$^+$ T cells (1/2000 to 1/200) responded rapidly to MBP by secreting interferon (IFN)-γ, whereas such a rapid T cell response to MBP could not be detected in the presence of NK cells. Nor did we detect the memory response to MBP in the 'CD95$^+$ NK-low' multiple sclerosis patients in remission or healthy subjects, regardless of whether NK cells were depleted or not. Depletion of cells expressing CD16, another NK cell marker, also caused IFN-γ secretion from MBP-reactive CD4$^+$ T cells in the PBMCs from 'CD95$^+$ NK-high' multiple sclerosis. Moreover, we showed that NK cells from 'CD95$^+$ NK-high' multiple sclerosis could inhibit the antigen-driven secretion of IFN-γ by autologous MBP-specific T cell clones *in vitro*. These results indicate that NK cells may regulate activation of autoimmune memory T cells in an antigen non-specific fashion to maintain the clinical remission in 'CD95$^+$ NK-high' multiple sclerosis patients.

Keywords: multiple sclerosis; myelin basic protein; NK cell; NK2; T cell–NK cell interaction

Abbreviations: CBA = cytokine bead array; HLA = human leukocyte antigen; IFN = interferon; IL = interleukin; MBP = myelin basic protein; MS-rel = multiple sclerosis in relapse; MS-rem = multiple sclerosis in remission; NK = natural killer; NK2 = NK type 2; OVA = ovalbumin; PBMCs = peripheral blood mononuclear cells; PI = propidium iodide; PLP = proteolipid protein; TCC = T-cell clone; TNF = tumour necrosis factor

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Introduction

Multiple sclerosis is a chronic neurological disease the pathology of which is characterized by multiple foci of inflammatory demyelinating lesions accompanying a variable degree of axonal changes (Bjartmar and Trapp, 2001). Regarding the pathogenesis of multiple sclerosis, studies have indicated that autoimmune T cells targeting myelin components play a crucial role in mediating the inflammatory process, particularly in the early stages of relapsing–remitting multiple sclerosis (Steinman, 2001). A number of laboratories have studied the properties of potentially pathogenic autoimmune T cell clones (TCC) reactive to myelin antigens such as myelin basic protein (MBP) and proteolipid protein (PLP), which have been derived from the peripheral blood of multiple sclerosis (Ota *et al.*, 1990; Pette *et al.*, 1990; Martin *et al.*, 1991; Ohashi *et al.*, 1995). The large majority of the TCC are CD4$^+$ and produce T helper type 1 (Th1) cytokines

such as interferon (IFN)-γ after recognizing the myelin peptide bound to human leukocyte antigen (HLA)-DR molecules. These results are consistent with the idea that the inflammatory process of multiple sclerosis is triggered by invasion of autoimmune Th1 cells into the CNS, and that exogenous or endogenous factors altering the Th1/Th2 balance may influence the disease activity. The relevance of this postulate is actually supported by clinical observations that Th2-inducing medications, such as copolymer-1, are beneficial for multiple sclerosis (Duda *et al.*, 2000; Neuhaus *et al.*, 2000), and that administration of IFN-γ showed deleterious effects on multiple sclerosis in previous clinical trials (Panitch *et al.*, 1987).

Although there are a number of candidate target antigens for multiple sclerosis, MBP is thought to be a primary target for autoimmune T cells, at least in some patients (Bielekova *et al.*, 2000). It is of note that MBP- or PLP-specific TCC can be established not only from multiple sclerosis, but also from peripheral blood of healthy subjects, which raised the intriguing issue as to how healthy subjects are protected from self-attack by the potentially pathogenic autoimmune Th1 cells. Although much remains to be clarified, studies in the last decade have showed that regulatory cells are involved in prevention of or recovery from autoimmune diseases in rodent (Das *et al.*, 1997; Zhang *et al.*, 1997; Olivares-Villagomez *et al.*, 1998; Sakaguchi *et al.*, 2001). This allows us to speculate that regulatory cells may contribute to protecting healthy subjects from developing autoimmune diseases such as multiple sclerosis, or to prohibiting acute attacks or enhancing the recovery from clinical exacerbations in patients with relapsing–remitting multiple sclerosis.

Whereas regulatory cells constitute various lymphoid populations, substantial evidence supports that natural killer (NK) cells play significant roles in protecting against autoimmune diseases (Zhang *et al.*, 1997; Matsumoto *et al.*, 1998; Smeltz *et al.*, 1999). In fact, it has previously been demonstrated that NK cell depletion augments the severity of a model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) (Zhang *et al.*, 1997; Matsumoto *et al.*, 1998), which can be induced by sensitization against CNS myelin component. Given that autoimmune Th1 cells would mediate the pathology of EAE, we propose a possible involvement of NK cells in suppressing autoimmune Th1 cells in multiple sclerosis.

With the hypothesis that NK cells may contribute to maintaining the remission in relapsing–remitting multiple sclerosis, we have previously examined the cytokine production and surface phenotype of NK cells freshly isolated from the peripheral blood mononuclear cells (PBMCs) of multiple sclerosis in remission (MS-rem) or relapse (MS-rel) (Takahashi *et al.*, 2001). The results demonstrate that NK cells in MS-rem (but not MS-rel) are characterized by a remarkable elevation of interleukin (IL)-5 mRNA and a decreased expression of IL-12Rβ2 mRNA, as well as a higher percentage of $CD95^+$ cells among the $CD56^+$ NK cells. These features of the cells are reminiscent of NK type 2 (NK2) cells, which can be induced *in vitro* in the presence of IL-4 and of anti-IL-12 antibodies (Peritt *et al.*, 1998). The NK2 cells induced from PBMCs of healthy subjects inhibit the generation of IFN-γ-secreting Th1 cells from the PBMCs of the same subjects (Takahashi *et al.*, 2001), leading us to postulate that NK2-like cells detected in MS-rem may play a regulatory role. While the NK2-like features were found to be lost in patients at acute relapsing state, they tended to be restored along with clinical recovery. Obviously, these results do not imply that clinically diagnosed MS-rem represents a homogeneous condition. In fact, the parameters characteristic for NK2-like cells (i.e. up-regulation of IL-5 mRNA and an increased frequency of $CD95^+$ cells) showed a substantial variance in MS-rem, indicating their heterogeneity.

More recently, we have noticed that MS-rem can be divided into two subgroups, '$CD95^+$ NK-high' and '$CD95^+$ NK-low', according to the frequency of $CD95^+$ cells among NK cells. Here, we demonstrate that these two groups significantly differ in the responsiveness to MBP *ex vivo* in an NK-cell-depleted condition. Namely, NK-deleted PBMCs from '$CD95^+$ NK-high' multiple sclerosis responded rapidly to MBP, as assessed by the frequency of IFN-γ-secreting $CD4^+$ T cells at 8 h after stimulation with MBP, whereas those from the '$CD95^+$ NK-low' or from healthy subjects responded only marginally. Moreover, we showed that NK cells from a '$CD95^+$ NK-high' multiple sclerosis could inhibit the antigen-driven secretion of IFN-γ by MBP-specific TCC established from the same patient. These results demonstrate, for the first time to our knowledge, that NK cell depletion leads to augmentation of memory T cell response to an autoantigen in human, and that an elaborate interplay between NK cells and MBP-specific memory T cells may be involved in the regulation of multiple sclerosis in '$CD95^+$ NK-high' patients.

Material and methods

Subjects

To clarify the heterogeneity among patients with MS-rem regarding NK cell phenotype, we first examined 30 patients with MS-rem (male/female = 11/19; aged 37.7 ± 11.1 years) for the lymphoid cell expression of CD95. As a control for multiple sclerosis, we examined 26 healthy sex- and age-matched subjects (male/female = 11/15; aged 39.9 ± 12.2 years). Furthermore, for a new cohort of 14 patients with MS-rem (male/female = four/10; aged 39.2 ± 10.7 years) (Table 1) and 14 healthy subjects (male/female = five/nine; aged 35.3 ± 8.0 years), we conducted the cytokine secretion assay as well as flow cytometer analysis for the frequency of $CD95^+$ NK cells. Two of the patients were examined again after a 1-year interval.

Written informed consent was obtained from all patients and healthy volunteers and the study was approved by the Ethics Committee of the National Center of Neuroscience (NCNP). All patients fulfilled standard criteria for the diagnosis of relapsing–remitting multiple sclerosis (Poser *et al.*, 1983; McDonald *et al.*, 2001). The clinical status of multiple sclerosis (MS-rem or MS-rel) was operationally determined as described previously (Takahashi *et al.*, 2001). In brief, we selected MS-rem patients for study who had been clinically stable without any immunosuppressive medications for >3 months, and had shown no sign of new lesions as assessed by a recent MRI scan with gadolinium enhancement. None of our patients represented the pure optic-spinal form of multiple sclerosis (Misu *et al.*, 2002), which may be rather unique to Japanese populations.

Table 1 *List of the PBMC samples examined for the frequency of memory Th1 cells*

Information on patients			
PBMC code	Age (years)/sex	$CD95^+$ NK frequency	EDSS#
#1	43/M	High	2.5
#2	30/F	High	2.5
#3	53/M	High	1.0
#4	39/F	High	3.5
#5	28/F	High	1.0
#6*	35/M	Low	2.0
#7**	57/F	Low	3.0
#8	31/M	Low	1.0
#9	29/F	Low	3.0
#10	38/F	Low	2.0
#11	59/F	High	3.5
#12*	36/M	High	2.0
#13**	58/F	High	3.0
#14	33/F	High	6.5
#15	29/F	Low	1.0
#16	45/F	Low	4.0

The samples marked with * or ** are derived from the same patients, with an interval of 1 year between samples. The phenotype of both of these patients changed from '$CD95^+$ NK-low' to '$CD95^+$ NK-high'. M = male; F = female; EDSS = Expanded Disability Status Scale.

Reagents

Anti-CD3-FITC or -ECD, anti-CD4-PC5, anti-CD8-FITC, anti-CD16-Phytoerythrin, and anti-CD56-PC5 or -PE mAbs were purchased from IMMUNOTECH (Marseille, France). Anti-CD57-FITC, anti-CD69-PE, anti-CD94-FITC, anti-CD95-FITC, -Cych or -PE, anti-CD158a-FITC, anti-NKB1-FITC, and anti-HLA-DR-FITC mAbs were purchased from BD PharMingen (San Jose, CA, USA). Human MBP was purified with a modification of previously described methods (Deibler *et al.*, 1972, 1995).

Cell preparation and NK cell deletion

Shortly after drawing peripheral blood, PBMCs were separated by density gradient centrifugation with Ficoll-Hypaque™ PLUS (Amersham Biosciences, Uppsala, Sweden). They were washed three times in phosphate-buffered saline (PBS), and resuspended at 1×10^6 cells/ml in AIM-V culture medium (Invitrogen Corp., Carlsbad, CA, USA) containing 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Rockville, MD, USA). NK cells were depleted from the PBMCs with either CD56- or CD16-MicroBeads (Miltenyi Biotech, Gradbach, Germany), following the protocol provided by the manufacturer.

T cell clones

$CD4^+$ TCC were generated from a '$CD95^+$ NK-high' multiple sclerosis patient (HLA-DRB1*1502) by repeated selection against human whole MBP with modification of a previously described method (Pette *et al.*, 1990). The TCC proliferated and secreted Th1 cytokines specifically in response to MBP, and the proliferative response and cytokine production was greatly reduced in the presence of antibodies against HLA-DR. The DR-restricted clone cells were grown in AIM-V medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

T-cell stimulation with MBP

To assess the presence of memory MBP-reactive T cells in the peripheral blood, fresh PBMCs or NK-deleted PBMCs were stimulated for 8 h with 10 μg/ml MBP in 96-well round-bottomed plates at 2×10^5 cells/well, and then analysed for the presence of IFN-γ-secreting cells using the cytokine secretion assay. To evaluate the regulatory function of NK cells from '$CD95^+$ NK-high' multiple sclerosis, resting cells of MBP-specific TCC (2×10^4 cells/well) were stimulated with 10 μg/ml MBP in the presence of X-irradiated (5000 rad) autologous total PBMCs or $CD56^+$ NK-deleted PBMCs (1×10^5 cells/well) for 8 h prior to the cytokine secretion assay, and for 60 h to determine the proliferation of the TCC. To assess cell proliferation, we counted incorporation of [^{3}H]thymidine (1 μCi/well) during the final 12 h with a beta-1205 counter (Pharmacia, Uppsala, Sweden).

Cytokine secretion assay

We used a commercial kit from Miltenyi Biotech to identify T cells secreting IFN-γ. The principle of this assay has been described previously (Manz *et al.*, 1995). Briefly, cells were stained with IFN-γ capture antibody 8 h after stimulation with MBP or ovalbumin (OVA), then washed and cultured again for 45 min. They were stained with PE-conjugated IFN-γ detection antibody, together with anti-human CD3-FITC and -CD4-PC5, then washed and resuspended in PBS containing propidium iodide (PI) (BD PharMingen). Samples were analysed using flow cytometry.

Cytokine bead array

The levels of IL-2, -4, -5, -10, tumour necrosis factor (TNF)-α and IFN-γ in the culture supernatants were measured by cytokine bead array (CBA) (BD PharMingen), in which six bead populations with distinct fluorescence intensities are coated with capture antibodies specific for each cytokine (Cook *et al.*, 2001). The cytokine capture beads were mixed with the PE-conjugated detection antibodies and then incubated with recombinant standards or supernatant samples to form sandwich complexes. After washing the beads, sample data were acquired using the flow cytometer and were analysed with the BD CBA Analysis Software™ (BD PharMingen).

Results

An increased frequency of $CD95^+$ NK cells distinguishes a subgroup of multiple sclerosis

As we have reported previously (Takahashi *et al.*, 2001), whereas proportions of $CD3^-$ $CD56^+$ NK cells in fresh PBMCs weakly express CD95 on their surface, the frequency of $CD95^+$ NK cells is significantly elevated in MS-rem as compared with healthy subjects or MS-rel. We have further noticed that MS-rem can be divided into two subgroups according to the frequency (%) of $CD95^+$ cells among NK cells (Fig. 1A; see also the left panels in Figs 1B and 2A, showing the distinction between $CD95^+$ and $CD95^-$ cells). When we determined the mean + 2 SD value for healthy subjects (35.86%) as an upper boundary for healthy subjects,

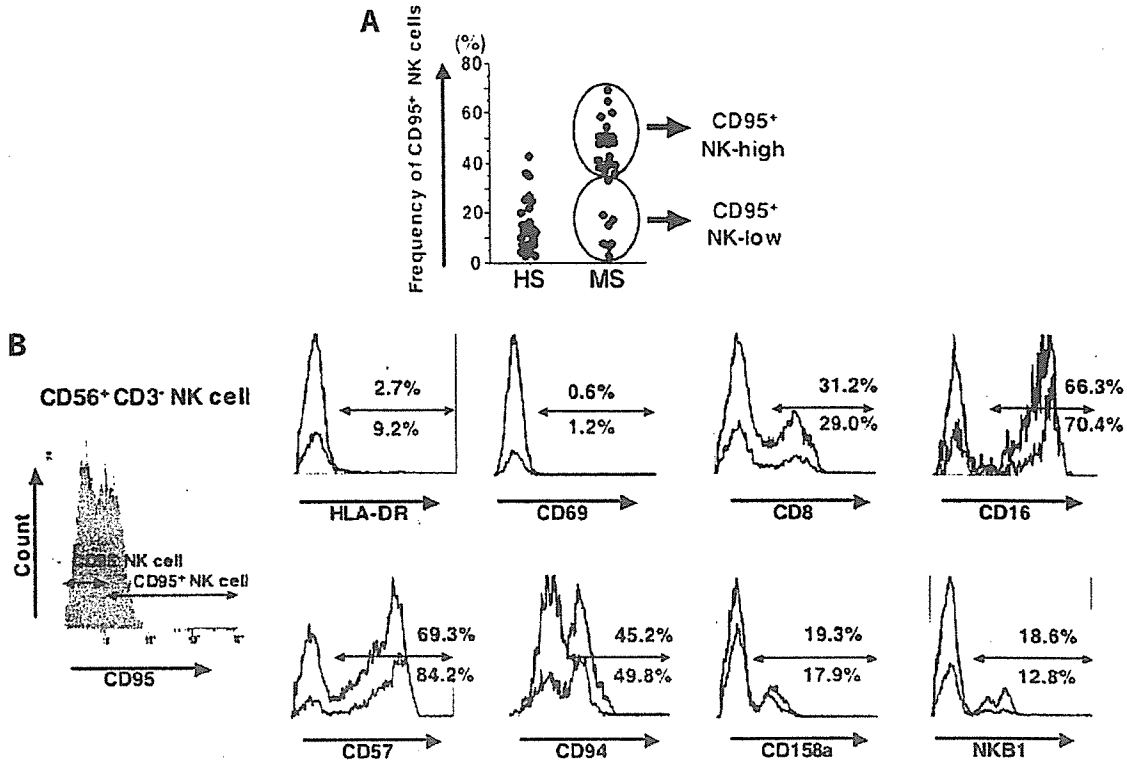

Fig. 1 Characterization of CD95$^-$ NK cells from 'CD95$^+$ NK-high' multiple sclerosis. (A) Multiple sclerosis patients in remission (MS-rem) can be subgrouped into 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low'. Freshly isolated PBMCs from 26 healthy subjects or 30 MS-rem were stained with the combination of anti-CD3-FITC, -CD56-PC5 and -CD95-PE, and evaluated for the frequency of CD95$^+$ cells in the CD56$^+$CD3$^-$ NK cell population (the fluorescence intensity for CD95 expression is shown in the histograms in B and Fig. 2A). Note that flow fluorocytometric analysis was completed within 2 h after drawing blood in order to avoid spontaneous death of CD95$^+$ cells. (B) Comparison of CD95$^+$ versus CD95$^-$ NK cells in the expression of various surface molecules. We stained the PBMCs from 'CD95$^+$ NK-high' patients with the panel of antibodies for surface molecules expressed by NK cells. Red lines represent the histogram gated for CD95$^-$ NK cells and blue lines for CD95$^+$ NK cells. Values in red and in blue represent the positive percentage in CD95$^-$ and CD95$^+$ cells, respectively. As indicated, CD95$^+$ NK cells did not differ significantly from CD95$^-$ NK cells in the staining pattern for each antibody regarding the proportion of positive cells as well as the mean fluorescence intensity. Shown are the results of a representative case.

three-quarters of MS-rem had a percentage value higher than this boundary. We defined these patients in remission with a higher frequency of CD95$^+$ cells in NK cells as 'CD95$^+$ NK-high' multiple sclerosis, and the rest as 'CD95$^+$ NK-low' (Fig. 1A). In contrast to CD56$^+$ NK cells, CD3$^+$CD56$^-$ T cells and CD3$^+$ CD56$^+$ NK T cells were not different between healthy subjects and multiple sclerosis patients as regards the frequency of CD95$^+$ cells (data not shown), which directed our attention to the analysis of CD56$^+$ NK cells.

Because NK cells from MS-rem were found to express a larger amount IL-5 mRNA, and since they were neither defective in cytolytic function nor reduced in number (Takahashi *et al.*, 2001), we hypothesized that the CD95 expression may reflect an activation state of the NK cells. To test this hypothesis, we compared the CD95$^+$ and CD95$^-$ NK populations derived from 'CD95$^+$ NK-high' patients by flow cytometry. Histogram plot analysis for the proportion of positive cells and for mean fluorescence intensity showed that the two populations are analogous in the expression of HLA-DR, CD69, CD8, CD16, CD57, CD94, CD158a and NKB1 (Fig. 1B). Whereas HLA-DR and CD69 molecules are regarded as cell activation markers, few populations of CD95$^+$ NK cells from multiple sclerosis or healthy subjects expressed these molecules. These results do not support the idea that the CD95$^+$ NK cells are in a state of activation, nor do they indicate that the CD95$^+$ cells represent a unique subset of monoclonal or oligoclonal origin. It has recently been suggested that CD56^{bright} NK cells may represent a distinct subset (Jacobs *et al.*, 2001). However, we saw no difference in the proportion of CD56^{bright} cells between CD95$^+$ and CD95$^-$ NK cells (data not shown).

CD56$^+$ NK cell depletion induces the rapid activation of MBP-reactive memory T cells in PBMCs from 'CD95$^+$ NK-high' multiple sclerosis

We have previously shown that the CD95$^+$ NK cells found in multiple sclerosis patients resemble the NK cells that can be induced in culture in the presence of IL-4 and anti-IL-12

mAb [referred to as 'NK2-like cells' according to the definition by Peritt *et al.* (1998)]. We also found that Peritt's NK2 cells induced *in vitro* inhibited the induction of IFN-γ-secreting T cells from peripheral T cells after stimulation with phorbol myristate acetate and ionomycin (Takahashi *et al.*, 2001). Based on these observations, we speculated that NK cells might prohibit Th1 cell activation in the remission of multiple sclerosis in an antigen-non-specific manner, and contribute to maintaining the remission. However, it remained an open question as to whether the NK2-like cells found in MS-rem would indeed regulate pathogenic autoimmune T cells *in vivo*. To investigate functions of NK cells in MS-rem, we evaluated the effect of NK cell depletion on the peripheral T cell response to MBP, a major target antigen of multiple sclerosis (Bielekova *et al.*, 2000). In brief, we depleted $CD56^+$ cells from the PBMCs with a magnetic sorter, and then stimulated the NK-deleted populations as well as whole PBMCs with MBP *in vitro* for 8–24 h. Subsequently, we detected the antigen-responsive T cells based on the secretion of IFN-γ (Manz *et al.*, 1995). The preparatory experiments revealed that 8 h of stimulation provides an optimal condition yielding a low background (0–0.03%). This novel assay enables us to selectively detect memory-type Th1 cells that can respond rapidly to antigen, whereas previous assays that depend on long-term cultures (Pette *et al.*, 1990; Martin *et al.*, 1992) evaluate not only memory but also naive T cells. Of note, there is a general consensus that peripheral blood of multiple sclerosis patients contains MBP-reactive T cells that are activated and/or differentiated into memory T cells (Allegretta *et al.*, 1990; Martin *et al.*, 1992; Zhang *et al.*, 1994; Lovett-Racke *et al.*, 1998; Scholz *et al.*, 1998).

We examined 16 PBMC samples from 14 MS-rem patients (nine samples from '$CD95^+$ NK-high', and seven from '$CD95^+$ NK-low') and 14 healthy subjects (see Table 1). When freshly isolated PBMCs were stimulated with MBP before NK cell depletion, four MS-rem and five healthy subjects samples showed a marginal response to MBP (0.01–0.03% increase of IFN-γ-positive cells among $CD4^+$ T cells). We did not find any significant response to MBP with the other PBMC samples. In contrast, when cells were stimulated with MBP after deleting $CD56^+$ NK cells, a significant response with a stimulatory index >3 was detected in seven of the nine '$CD95^+$ NK-high' samples, and a marginal response was detected in two (Fig. 2A and B). Of note, none of the NK-deleted samples from the '$CD95^+$ NK-low' patients and healthy subjects showed a definitive response to MBP. The difference for the '$CD95^+$ NK-high' versus the '$CD95^+$ NK-low' or healthy subjects was statistically significant (Fig. 2B). These *ex vivo* experiments have revealed that the '$CD95^+$ NK-high' patients may possess a higher number of T cells that can rapidly respond to MBP (MBP-specific memory T cells), compared with '$CD95^+$ NK-low' MS-rem or healthy subjects. In other words, they provide strong evidence for clonal expansion of memory autopathogenic T cells in the '$CD95^+$ NK-high' patients. However, as we could demonstrate an increase of the memory autoimmune T cells only after depleting NK cells, we interpreted that the potentially hazardous autoimmune T cells are being controlled by counter-regulatory NK cells in the '$CD95^+$ NK-high' patients. Of note, previous studies relying on alternative assays have revealed the presence of MBP-reactive T cells with activated and/or memory phenotypes at similar high frequencies in not all, but a major portion, of multiple sclerosis patients (Allegretta *et al.*, 1990; Zhang *et al.*, 1994; Bieganowska *et al.*, 1997; Lovett-Racke *et al.*, 1998; Scholz *et al.*, 1998; Illés *et al.*, 1999).

We conducted the same assay with a foreign antigen OVA in three of the '$CD95^+$ NK-high' (PBMC codes #3, #4 and #5 in Table 1) and one of the '$CD95^+$ NK-low' samples (#6). However, OVA-reactive T cells could not be detected in any sample of the fresh or NK-deleted PBMCs (data not shown). Because NK cells cannot discriminate T cells with different antigen specificities, the negative response to OVA in the four multiple sclerosis patients was interpreted to mean that they do not possess clonally expanded memory T cells reactive to OVA.

Depletion of $CD16^+$ NK cells also allows detection of MBP-reactive memory T cells in PBMCs from '$CD95^+$ NK-high' multiple sclerosis

Although we used anti-CD56 magnetic beads to deplete NK cells in the above experiments, the method would also deplete $CD3^+CD56^+$ NK T cells that may possibly play a role in the regulation of autoimmunity. To evaluate the possible contribution of $CD3^+CD56^+$ NK T cells, we next depleted NK cells from PBMCs from two '$CD95^+$ NK-high' patients on the basis of their expression of CD16. We found that after treatment with CD16-MicroBeads, almost all of $CD56^+$ NK cells are deleted, but $CD56^+CD3^+$ NKT cells remain largely untouched (Fig. 3A). However, like $CD56^+$-cell-deleted PBMCs, the $CD16^+$-cell-deleted PBMCs responded to MBP, as assessed by the induction of IFN-γ-secreting $CD4^+$ T cells (Fig. 3B). The responses found in the two patients were considered significant with regard to both percentage increase of IFN-γ-secreting cells (0.08% and 0.04%) and the stimulatory index (9.0 and 5.0) obtained after MBP stimulation. This result indicates that responsible cells to regulate autoimmune T cells in '$CD95^+$ NK-high' multiple sclerosis are not $CD56^+CD3^+$ NK T cells but NK cells.

Unfortunately, it remains unclear whether only $CD95^+$ NK cells play a regulatory role in '$CD95^+$ NK-high' multiple sclerosis or whether $CD95^-$ cells could also exhibit regulatory functions in the patients. We attempted to compare directly the function of $CD95^+$ and $CD95^-$ populations. However, isolation of $CD95^+$ NK cells with a cell sorter invariably induced cell activation as revealed by the expression of various activation markers. Furthermore, the isolated cells tended to die rapidly, probably due to CD95 ligation by the antibody (data not shown).

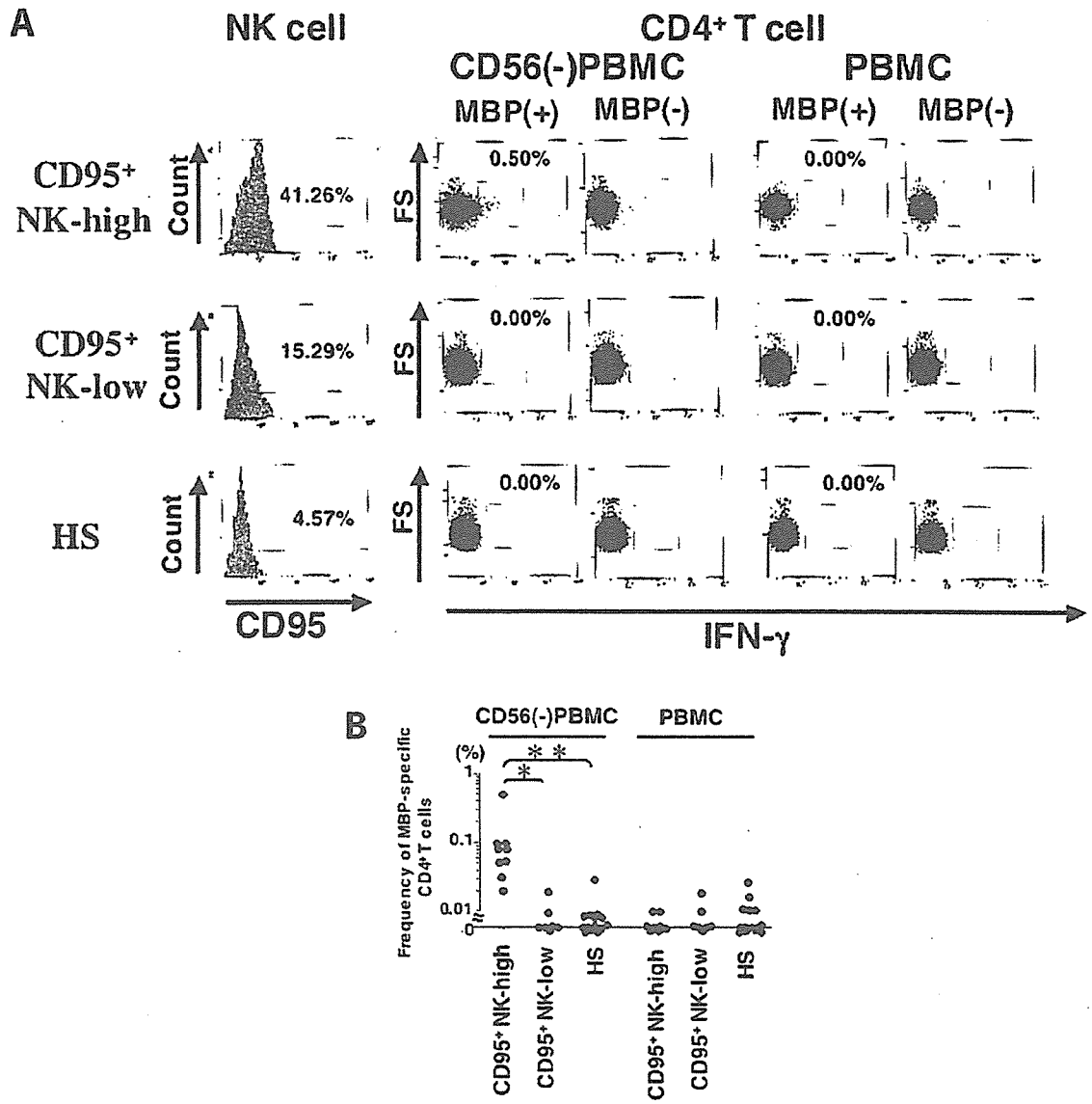

Fig. 2 Evidence for the role of NK cells in the regulation of MBP-reactive memory T cells in 'CD95$^+$ NK-high' multiple sclerosis. (**A**) IFN-γ secretion assay for NK-cell-deleted PBMCs and freshly isolated PBMCs. Whole PBMCs or PBMCs depleted for CD56$^+$ NK cells [CD56(−) PBMC] from the 'CD95$^+$ NK-high' multiple sclerosis (n = 9), 'CD95$^+$ NK-low' multiple sclerosis (n = 7) or healthy subjects (n = 14) were stimulated with 10 μg/ml of human MBP for 8 h for the IFN-γ secretion assay. The cells were also stained with anti-CD4-PC5 and -CD3-FITC, and the CD4$^+$CD3$^-$ and PI$^-$ cells were gated for analysis. Here we show representative results from 'CD95$^+$ NK-high' (*top*), 'CD95$^+$ NK-low' (*middle*) and healthy subjects (*bottom*). The IFN-γ-secreting CD4$^+$ T cells are shown as red dots; blue dots represent IFN-γ-negative cells. The histograms demonstrate the level of CD95 expression on the fresh CD56$^+$ NK cells from each individual, and the attached values show the frequency of CD95$^+$ cells. (**B**) Frequency (%) of MBP-reactive memory T cells among CD4$^+$ T cells. By using the cytokine secretion assay, we determined the frequency of IFN-γ-positive cells among CD4$^+$ T cells in each individual after culture with or without MBP. Here we plot the Δ% values [(%) with MBP − (%) without MBP], which represent the frequency of MBP-reactive CD4$^+$ T cells in each subject. Kruskal–Wallis test with Scheffe's F *post hoc* test was used for statistical analysis. *$P < 0.05$; **$P < 0.02$.

NK cells from 'CD95$^+$ NK-high' multiple sclerosis inhibit IFN-γ production by MBP-reactive T cell clones

To analyse how the NK cells from 'CD95$^+$ NK-high' multiple sclerosis efficiently control autoimmune T cell responses, we established three MBP-specific TCC from a 'CD95$^+$ NK-high' patient. These TCC proliferated and secreted IFN-γ, TNF-α, IL-2 and IL-5 in response to MBP presented by irradiated, fresh autologous PBMCs. Using the proliferation response and cytokine secretion by

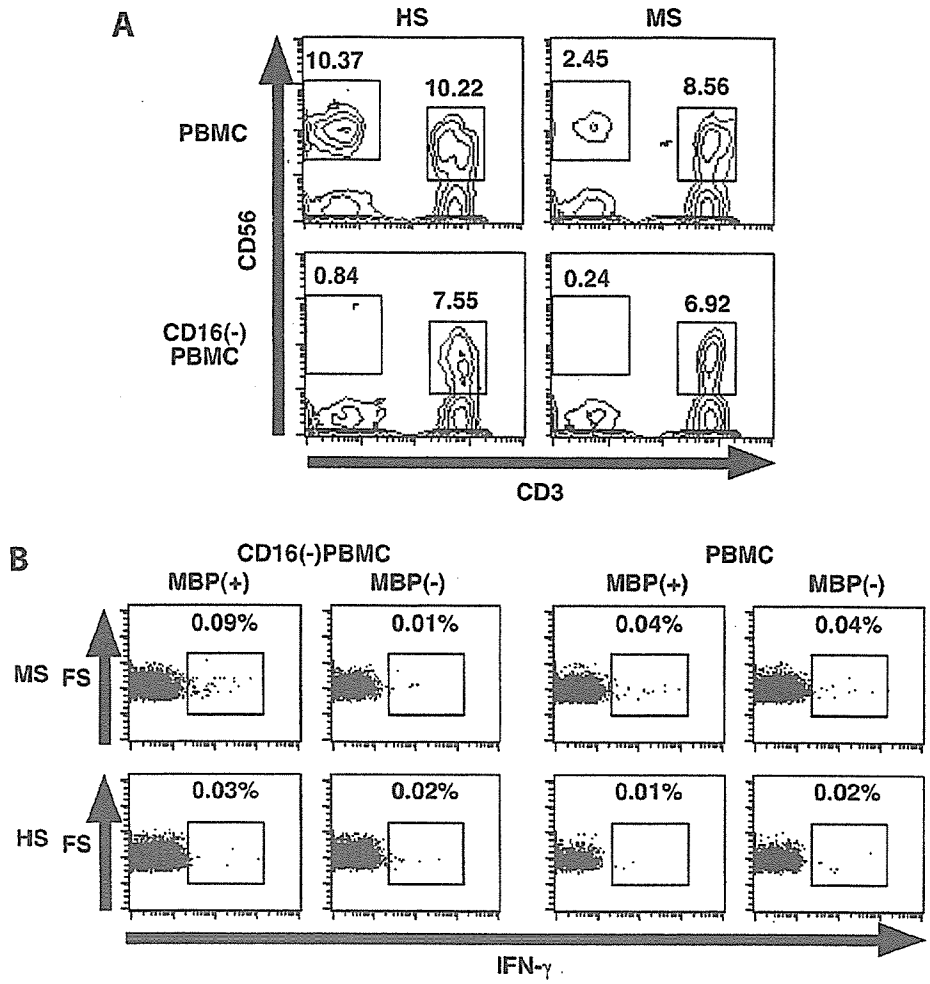

Fig. 3 Depletion of CD16$^+$ cells also allows detection of MBP-specific memory T cells in 'CD95$^+$ NK-high' multiple sclerosis. (**A**) Changes in the frequency of CD56$^+$ NK cells and CD56$^+$ NKT cells after deleting CD16$^+$ cells. Using CD16 microbeads, we deleted CD16$^+$ cells from PBMCs from two 'CD95$^+$ NK-high' patients and from two healthy subjects. The cells were stained with anti-human CD3-FITC and anti-CD56-PC5 to check the proportion of CD56$^+$ NK cells and CD56$^+$ T cells before and after CD16$^+$ cell depletion (upper versus lower panels). Shown are the results of a representative pair of multiple sclerosis and healthy subjects. (**B**) CD16$^+$-cell-depleted PBMCs from 'CD95$^+$ NK-high' multiple sclerosis responded rapidly to MBP. Using the same PBMC samples (CD16$^+$ or CD16$^-$), we conducted the IFN-γ secretion assay as described in Fig. 2A. This figure shows the result of the representative pair of multiple sclerosis patients and healthy subjects.

the TCC as read-out, we compared the whole PBMCs and the NK cell-deleted PBMCs for the ability to present whole MBP to the autologous TCC. We found that the whole PBMCs did not differ from the NK-deleted PBMCs in the ability to induce MBP-driven proliferation of TCC (Fig. 4A). However, the proportion of IFN-γ-secreting T cells among the TCC increased significantly when the NK cell-depleted PBMCs were used as antigen presenting cells (APC) (Fig. 4B). We also noticed a significant elevation of IFN-γ in the culture supernatant along with the increase of IFN-γ-secreting T cells (Fig. 4C). However, neither TNF-α nor IL-2 production was enhanced by NK cell depletion. These results support the view that NK cells from 'CD95$^+$ NK-high' multiple sclerosis regulate autoimmune T cells by inhibiting the T cell production of IFN-γ.

Discussion

It is generally held that relapse of multiple sclerosis represents the destructive CNS inflammation triggered by recently activated autoimmune T cells. In other words, pathogenic autoimmunity is apparently active during clinical relapse, which can be objectively defined by clinical status as well as MRI findings. In contrast, remission of multiple sclerosis, which is chiefly determined by exclusion of active inflammation in the CNS, may probably cover a wider range of disease states.

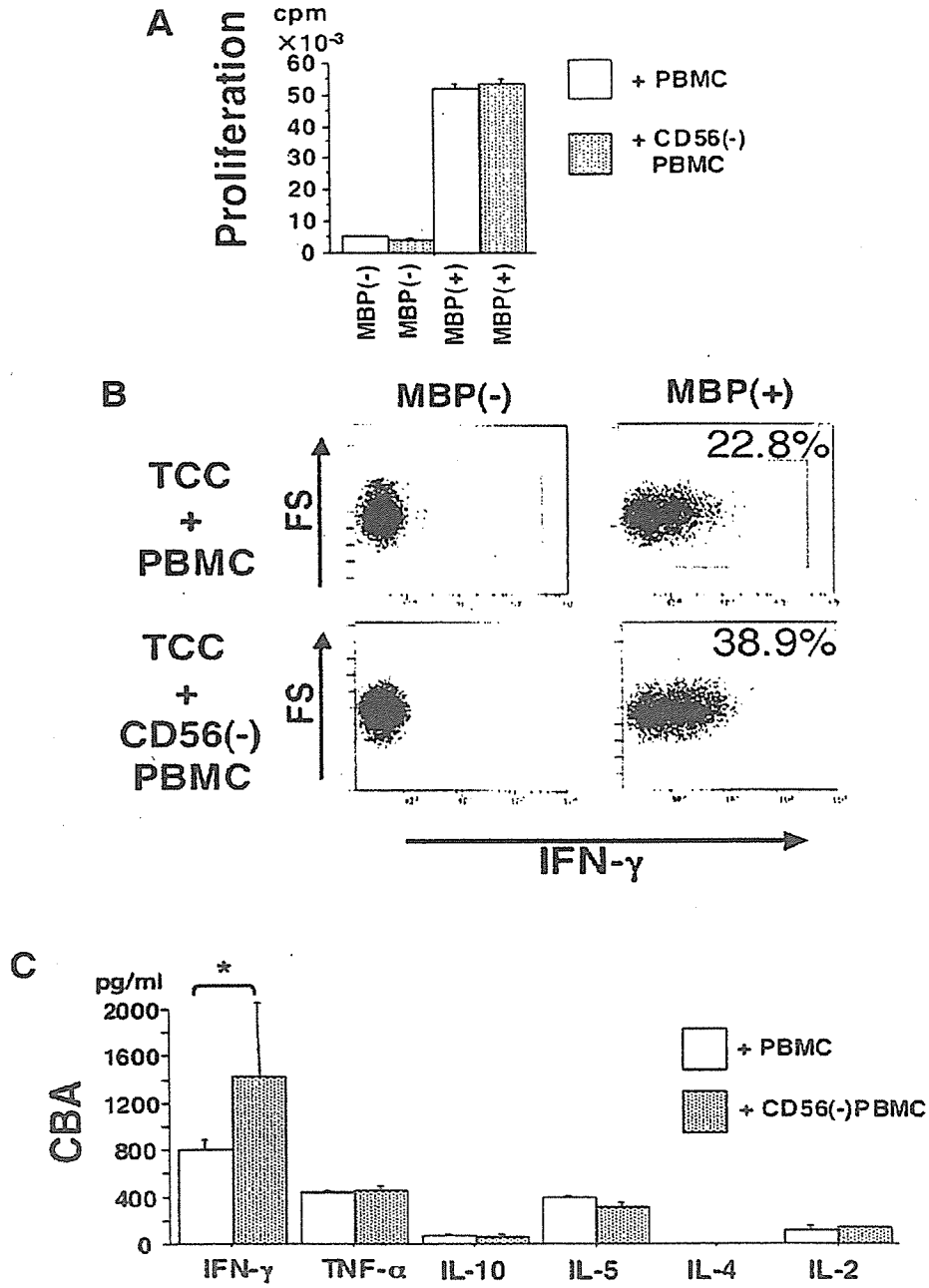

Fig. 4 Depletion of NK cells augments the antigen-presenting potential of PBMCs from 'CD95$^+$ NK-high' multiple sclerosis. (A) Effect of NK-cell deletion on the proliferation of MBP-specific TCC. We established three MBP-specific TCC from a 'CD95$^+$ NK-high' patient, and evaluated the proliferative response of the clone cells to MBP (10 μg/ml) in the presence of fresh autologous PBMCs [+ PBMC] or NK-deleted PBMCs [+ CD56 (−) PBMC]. This is a representative result of three TCC, which yielded essentially the same results. Data represent mean ± SD of quadruplicate cultures. (B) Effect of NK cell deletion on IFN-γ secretion by the MBP-specific TCC. MBP-specific TCC were cultured with or without MBP for 8 h in the presence of autologous PBMCs (upper panels) or of the autologous PBMCs depleted for CD56$^+$ NK cells (lower panels). We then conducted the cytokine secretion assay to detect IFN-γ-positive cells. Red dots indicate IFN-γ-secreting cells among CD4$^+$ CD3$^+$ PI$^-$ cells; blue dots represent IFN-γ-negative CD4$^+$ CD3$^+$ PI$^-$ T cells. The values (%) represent the frequency of IFN-γ-secreting cells among CD4$^+$ CD3$^+$ PI$^-$ cells. We conducted the assay with three TCC, which yielded essentially the same results. FS = forward scatter. (C) Effect of NK cell depletion on cytokine release by TCC into culture medium. The TCC were stimulated with MBP for 48 h in the presence of autologous PBMCs or NK-depleted PBMCs. Then we measured the concentrations of IFN-γ, TNF-α, IL-10, IL-5, IL-4 and IL-2 in the supernatants, using ELISA and CBA. Both assays yielded essentially the same results, and here we show the result of a CBA assay. Data represent mean ± SD. The Mann–Whitney *U*-test was used for statistical analysis. $^*P < 0.05$. We conducted the assay with three TCC, which yielded essentially the same results.

The present results show that multiple sclerosis patients in remission can be divided at least into two subgroups, 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low', based on the frequency of CD95$^+$ cells among NK cells. Furthermore, our functional analysis combining NK cell deletion and stimulation with MBP has indicated that the two subgroups differ significantly with regard to the responsiveness of the MBP-specific memory T cells to MBP in the absence of NK cells. Namely, after deleting CD56$^+$ NK cells, we saw a rapid induction of IFN-γ-secreting, anti-MBP T cells in 'CD95$^+$ NK-high' multiple sclerosis, whereas such a rapid response to MBP was not seen in 'CD95$^+$ NK-low' multiple sclerosis or healthy subjects. This result is in harmony with the previous results that clonally expanded MBP-specific T cells can be detected in a majority of multiple sclerosis patients (Zhang *et al.*, 1994; Smeltz *et al.*, 1999), and indicates that patients with an increased number of the autoimmune T cells may have the 'CD95$^+$ NK-high' phenotype during remission. Thus, the frequency of CD95$^+$ NK cells correlates with the frequency of MBP-reactive memory T cells and may serve as a useful marker to evaluate the immunological status of multiple sclerosis during remission.

The role of NK cells in the regulation of MBP-specific T cells was further strengthened by the demonstration that deletion of CD16$^+$ cells also enabled detection of memory MBP-specific T cells. Because we confirmed that depletion of the CD16$^+$ cells would greatly reduce the number of NK cells but did not significantly reduce CD56$^+$CD3$^+$ NK T cells, the role of the NK T cells in the regulation was excluded.

We have previously described that the 'CD95$^+$ NK-low' phenotype could also be seen in multiple sclerosis patients during relapse. However, the 'CD95$^+$ NK-low' phenotype in MS-rel was not persistent, but the 'CD95$^+$ NK-high' phenotype could be regained in a month or so along with clinical recovery. This fact raised the possibility that 'CD95$^+$ NK-low' MS-rem may represent an active state of multiple sclerosis, contrary to our speculation. To evaluate this possibility, we examined three patients with MS-rem for the 'CD95$^+$ NK-high/low' phenotype every 4–6 weeks, and found that they maintained the 'CD95$^+$ NK-low' phenotype for longer than several months (data not shown). This is in a striking contrast to the transient appearance of the 'CD95$^+$ NK-low' phenotype during relapse. Together with the clinical observations that these patients were in a very stable condition with minimal neurological disability, we estimate the disease condition in 'CD95$^+$ NK-low' MS-rem to be truly inactive and distinct from MS-rel.

It is of note that IFN-γ-secreting T cells could be identified as early as 8 h after stimulation with MBP in the absence of NK cells. This result implies that the NK cells should interact with the autoimmune T cells shortly after antigen stimulation to regulate very early T cell response. To account for such a rapid regulation by NK cells, we speculate that the regulatory NK cells may detect the subtle change of the autoimmune T cells during the early stage of activation. At present, very little is known about the molecular basis of T cell–NK cell interaction. However, it is obvious that NK cells must interact with T cells in an antigen-non-specific fashion, as they do not express highly variable receptors like T cell antigen receptors. Our results indicate that attempts to identify the ligand and receptors involved in T cell–NK interactions are very rewarding.

It is currently speculated that activation of autoimmune T cells could occur in response to microbial proteins whose sequence has a significant homology to the self-peptide (Steinman, 2001). We predict that the increased MBP-reactive Th1 cells in the 'CD95$^+$ NK-high' patients will most likely respond to microbial peptides mimicking MBP from time to time. However, counter-regulatory NK cells would maintain the clinical silence by actively suppressing activation of the autoimmune T cells that might lead to destructive CNS inflammation (Fig. 5). We then imagine that the clinical silence in the 'CD95$^+$ NK-high' patients could readily be disrupted when NK cells are numerically or functionally altered by exogenous or endogenous factors independent of multiple sclerosis (Wu *et al.*, 2000). In contrast, the clinical remission in 'CD95$^+$ NK-low' multiple sclerosis appears to be stable, as they are expected to possess much lower numbers of MBP-specific memory T cells, which does not necessitate the active

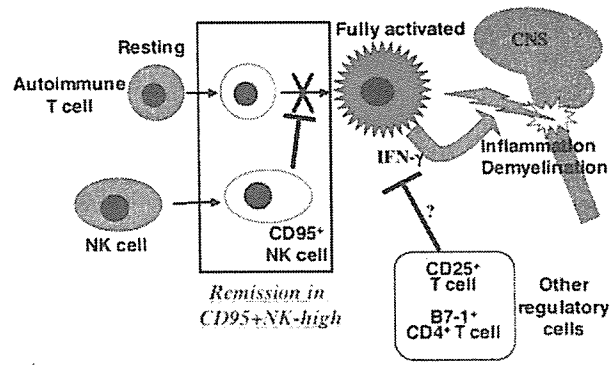

Fig. 5 The role of NK cells in 'CD95$^+$ NK-high' multiple sclerosis. As described in the text, the 'CD95$^+$ NK-high' patients are characterized by a concurrent increase of memory autoimmune T cells and CD95$^+$ NK cells. In the sense that memory autoimmune T cells cannot be detected in other patients in remission ('CD95$^+$ NK-low') even after NK cell depletion, we describe the immunological status of the 'CD95$^+$ NK-high' as a 'smouldering' state rather than 'remission'. Given that T cell recognition is much more promiscuous than previously anticipated, we imagine that autoimmune T cells in the 'CD95$^+$ NK-high' patients would respond to exogenous self-mimicking peptides from time to time. However, our results indicate that the CD95$^+$ NK cells could detect the early sign of T cell activation and then interact with autoimmune T cells to prohibit their full activation. Once this delicate control by NK cells is disrupted, the autoimmune T cells could be fully activated in response to the self-mimicking peptides. The fully activated T cells may be controlled by other regulatory cells such as CD4$^+$CD25$^+$ T cells (Sakaguchi *et al.*, 2001) or B7-1$^+$CD4$^+$ T cells (Kipp *et al.*, 2000). However, it is difficult to predict how efficiently the regulatory T cells may control the activated autoimmune T cells in individual cases.

engagement of regulatory NK cells. If these premises hold true, we may consider that the 'CD95$^+$ NK-high' patients are at a greater risk than 'CD95$^+$ NK-low' of developing relapses when exposed to potentially dangerous microbes that have cross-reactive epitopes. To describe the immunological status in 'CD95$^+$ NK-high', which seems to be more active than the 'CD95$^+$ NK-low', it might be appropriate to use the term 'smouldering' state rather than 'remission'.

After determining the presence of the 'CD95$^+$ NK-high' and 'CD95$^+$ NK-low' phenotypes in the patients with MS-rem, an important question might be whether the 'CD95$^+$ NK-high/-low' phenotype correlates with some clinical parameters or disease course. We speculated that 'CD95$^+$ NK-low' might be clinically less active than 'CD95$^+$ NK-high', when evaluated retrospectively. However, it might take time and would require a large number of patients to verify this postulate, taking the heterogeneity and chronic nature of the illness into consideration. Furthermore, it is of note that the 'CD95$^+$ NK-high' or '-low' phenotype appears to be interchangeable. For example, two of the patients who were examined for the memory T cell frequency showed the 'CD95$^+$ NK-low' phenotype in the first examination, but were found to have the 'CD95$^+$ NK-high' phenotype when examined 1 year later (Table 1). The phenotype switch in these patients was associated with an increase in the frequency of MBP-reactive memory T cells. We speculate that activity of multiple sclerosis may have been increased in these patients during the 1-year interval, although it is too early to draw any conclusions from the analysis of two patients.

Conversely, we have recently seen an opposing phenotype switch (from the 'CD95$^+$ NK-high' to 'CD95$^+$ NK-low') in two other patients. The frequency of CD95$^+$ cells among NK cells was >46.0% in both cases in the initial examinations, but the latest test showed normal values (27.4% and 10.0%). Although the patients appeared to be in the state of remission at the last examination, they developed serious signs of acute exacerbation 2 days later. As stated above, a transient switch from 'CD95$^+$ NK-high' to 'CD95$^+$ NK-low' could occur during relapse. Therefore, we speculate that the phenotype switch from 'high' to 'low' may be triggered by the very early events leading to clinical relapse. However, it is also possible that the reduction of the CD95$^+$ NK cells might have been triggered by multiple sclerosis-independent factors, such as infection or stress, and that this led to the occurrence of the relapse in these patients. This speculation is supported by the fact that a number of physiological conditions can alter NK cell number and/or function, and that CD95$^+$ NK cells tend do die more rapidly in culture than CD95$^-$ NK cells (our unpublished data). In future, it will be worthwhile to examine more systematically whether the phenotype switch may be the earliest marker to detect occurrence of relapse.

As Japanese neurologists have traditionally stressed that multiple sclerosis in Japan might be quite unique in immunopathology, it is theoretically possible that the regulatory function of CD95$^+$ NK cells reflects the uniqueness of Japanese multiple sclerosis and that the T cell–NK cell interaction is not operative in Caucasian multiple sclerosis. However, recent studies suggest that the frequency of pure optic-spinal form of multiple sclerosis linked with Japanese patients (Misu *et al.*, 2002) is drastically declining, possibly due to change in life-style or environmental factors in Japan (Yamamura, 2002; Houzen *et al.*, 2003). Reflecting this fact, the patients randomly recruited in this study did not have optic-spinal multiple sclerosis, and all had brain lesions similar to those found in Western multiple sclerosis. We therefore speculate that our experimental results will be reproduced in Caucasian patients in the future.

In summary, we have revealed that multiple sclerosis patients in remission have either 'CD95$^+$ NK-high' or 'CD95$^+$ NK-low' phenotype, and that 'CD95$^+$ NK-high' patients have a higher frequency of memory autoimmune T cells and have more active multiple sclerosis than 'CD95$^+$ NK-low' patients. Our *ex vivo* assay has demonstrated that 'CD95$^+$ NK-high' patients possess NK cells that actively inhibit activation of memory autoimmune T cells. In the sense that clinical silence depends on the functional regulatory NK cells, the condition of 'CD95$^+$ NK-high' is thought to be so unstable, as could be expressed by the term 'smouldering'. As such, evaluation of the NK cell functions and phenotypes in multiple sclerosis gives us a new insight into the autoimmune pathogenesis of multiple sclerosis, encouraging further efforts to clarify the NK cell–T cell interactions.

Acknowledgements

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The 14-3-3 Protein ϵ Isoform Expressed in Reactive Astrocytes in Demyelinating Lesions of Multiple Sclerosis Binds to Vimentin and Glial Fibrillary Acidic Protein in Cultured Human Astrocytes

Jun-ichi Satoh,* Takashi Yamamura,* and Kunimasa Arima†

From the Department of Immunology, National Institute of Neuroscience, and the Department of Neuropathology,† National Center Hospital for Mental, Nervous, and Muscular Disorders, National Center of Neurology and Psychiatry, Tokyo, Japan*

The 14-3-3 protein family consists of acidic 30-kd proteins expressed at high levels in neurons of the central nervous system. Seven isoforms form a dimeric complex that acts as a molecular chaperone that interacts with key signaling components. Recent studies indicated that the 14-3-3 protein identified in the cerebrospinal fluid of various neurological diseases including multiple sclerosis (MS) is a marker for extensive brain destruction. However, it remains unknown whether the 14-3-3 protein plays an active role in the pathological process of MS. To investigate the differential expression of seven 14-3-3 isoforms in MS lesions, brain tissues of four progressive cases were immunolabeled with a panel of isoform-specific antibodies. Reactive astrocytes in chronic demyelinating lesions intensely expressed β, ϵ, ζ, η, and σ isoforms, among which the ϵ isoform is a highly specific marker for reactive astrocytes. Furthermore, protein overlay, mass spectrometry, immunoprecipitation, and double-immunolabeling analysis showed that the 14-3-3 protein interacts with both vimentin and glial fibrillary acidic protein in cultured human astrocytes. These results suggest that the 14-3-3 protein plays an organizing role in the intermediate filament network in reactive astrocytes at the site of demyelinating lesions in MS. ***(Am J Pathol 2004, 165:577–592)***

The 14-3-3 protein family consists of evolutionarily conserved, acidic 30-kd proteins originally identified by two dimensional analysis of brain protein extract.[1–4] Seven isoforms of the 14-3-3 protein named β, γ, ϵ, ζ, η, θ (also termed as τ), and σ have been identified in eukaryotic cells. Although the 14-3-3 protein is widely distributed in neural and nonneural tissues, it is expressed most abundantly in neurons in the central nervous system (CNS), where it represents 1% of total cytosolic proteins.[4–7] A homodimeric or heterodimeric complex, which is composed of the same or distinct isoforms of the 14-3-3 protein, constitutes a large cup-like structure with two ligand-binding sites in its groove. The dimeric complex acts as a novel molecular chaperone that interacts with key molecules involved in cell differentiation, proliferation, transformation, and apoptosis.[1–4] It regulates the function of target proteins by restricting their subcellular location, bridging them to modulate catalytic activity, and protecting them from dephosphorylation or proteolysis.[1–4,8–10] In general, the 14-3-3 protein binds to phosphoserine-containing motifs of the ligands such as RSXpSXP and RXY/FXpSXP in a sequence-specific manner.[1–3,10] More than 100 proteins have been identified as being 14-3-3 binding partners, including a range of intracellular signaling regulators such as Raf, BAD, protein kinase C (PKC), phophatidylinositol 3-kinase (PI3K), and cdc25 phosphatase.[1–4,8–10] Binding of the 14-3-3 protein to Raf is indispensable for Raf kinase activity in the Ras/MAPK signaling pathway, whereas 14-3-3 binding to the mitochondrial Bcl-2 family member BAD, when phosphorylated by a serine/threonine kinase Akt, inhibits apoptosis.[1–4] In addition to the phosphorylation-dependent interaction, the 14-3-3 protein can interact with a set of target proteins in a phosphorylation-independent manner.[10–12] The ϵ isoform binds to p190RhoGEF via a phosphoserine-independent interaction.[11]

Previous studies indicated that the 14-3-3 protein has isoform-specific and nonredundant functions.[1–4] Synaptic transmission and associative learning are impaired in

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During submission of the present manuscript, an immunohistochemical study (Kawamoto Y, Akiguchi I, Kovács GG, Flicker H, Budka H: Increased 14-3-3 immunoreactivity in glial elements in patients with multiple sclerosis. Acta Neuropathol 2004, 107:137–143) has been published. This study showed that the 14-3-3 protein is expressed strongly in both astrocytes and oligodendrocytes in MS brains using an anti-14-3-3 protein antibody broadly reactive against all isoforms (H-8, sc-1657; Santa Cruz Biotechnology).

Supplemental information can be found on *http://www.amjpathol.org*.

Address reprint requests to Dr. Jun-ichi Satoh, Department of Immunology, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. E-mail: satoj@ncnp.go.jp.

Drosophila mutants lacking the ζ protein.[13] The 14-3-3 isoforms have distinct affinities for their target proteins. A preferential interaction is observed between PKCθ and the human 14-3-3θ isoform in T cells,[14] IGF1-receptor, IRS1, and ε isoform,[15] the apoptosis-inhibitor A20 and the human β and η isoforms,[16] and glucocorticoid receptor and the human η isoform.[17] The human β and ζ isoforms and not γ or ε isoforms interact with phosphorylated tau.[18] Furthermore, different isoforms show distinct patterns of spatial, temporal, and subcellular distribution. The human θ and σ isoforms are predominantly expressed in T cells and epithelial cells, respectively.[14,19] The rat ε and γ isoforms are enriched in the synaptosomal membranes.[20] and the γ isoform is the main 14-3-3 protein located in the Golgi apparatus in mammalian cells.[3] In the developing rat brain, defined populations of neurons express β, γ, ζ, and θ isoforms at specific stages of development.[6,7] In the adult mouse brain, β, γ, η, and ζ isoforms are widely distributed with the localization primarily in neurons, although some glial cells express ε, θ, and ζ isoforms.[21]

Recently, several lines of evidence have indicated that the 14-3-3 protein is involved in neurodegenerative processes. The 14-3-3 protein detected in the cerebrospinal fluid of Creutzfeldt-Jacob disease has been used as a biochemical marker for the premortem diagnosis of Creutzfeldt-Jacob disease in the context of differential diagnosis of progressive dementia.[22–24] In addition, intense immunoreactivity against the ζ isoform was identified in amyloid plaques in the Creutzfeldt-Jacob disease brain.[25] However, several studies including our own showed that the 14-3-3 protein is occasionally detectable in the cerebrospinal fluid of infectious meningoencephalitis, metabolic encephalopathy, cerebrovascular diseases, and multiple sclerosis (MS) presenting with severe myelitis, suggesting that it is not a marker specific for prion diseases but for extensive destruction of brain tissues causing the leakage of 14-3-3 protein into the cerebrospinal fluid.[4,22,26,27] In the Alzheimer's disease brain, neurofibrillary tangles express immunoreactivity against the 14-3-3 protein.[28] The 14-3-3ζ homodimer interacts with tau and glycogen synthase kinase-3β (GSK3β), and stimulates GSK3β-mediated tau phosphorylation.[29] In the Parkinson's disease brain, Lewy bodies possess γ, ε, ζ, and θ isoforms that interact with α-synuclein.[30,31] Dopamine-dependent neurotoxicity is mediated by a soluble complex composed of the 14-3-3 protein and α-synuclein, whose levels are markedly elevated in the substantia nigra of the Parkinson's disease brain.[32] The neurotoxicity of ataxin-1, the causative protein of spinocerebellar ataxia type 1, is enhanced by ε and ζ isoforms that bind to and stabilize ataxin-1 phosphorylated by Akt, thereby slowing its degradation.[33] Finally, expression of the θ isoform is enhanced in the spinal cord of amyotrophic lateral sclerosis.[34] However, it remains unknown whether the 14-3-3 protein plays an active role in the pathological process of MS.

In the present study, we investigated the differential expression of seven 14-3-3 isoforms in chronic active demyelinating lesions of MS. We found that reactive astrocytes intensely express β, ε, ζ, η, and σ isoforms, among which the ε isoform provides a specific marker to identify reactive astrocytes in the MS brain. Furthermore, the 14-3-3 protein interacts with vimentin and glial fibrillary acidic protein (GFAP) in cultured human astrocytes. These observations suggest that the 14-3-3 protein plays an organizing role in the intermediate filament (IF) network in reactive astrocytes at the site of demyelinating lesions in MS.

Materials and Methods

MS and Non-MS Brain Tissues

Ten-μ-thick tissue sections were prepared from the brain, spinal cord, and optic nerve derived from four autopsy cases of MS numbered 791, 744, 609, and 544. The clinical and neuroradiological profiles of these patients are shown in a supplementary table on The American

Table 1. The 14-3-3 Isoform-Specific or Broadly Reactive Antibodies Utilized for Immunochemistry and Western Blot Analysis

14-3-3 isoforms	Suppliers	Code	Antigen peptide	Origin	Specificity	Concentration used for immunohistiochemistry (μg/ml)	Concentration used for Western blotting (μg/ml)
Pan	SC	sc-629	N-terminal	Rabbit	Reactive to all isoforms	0.4	0.04
Pan	SC	sc-1657	N-terminal	Mouse	Reactive to all isoforms	0.4	0.04
β	SC	sc-626	C-terminal	Rabbit	Reactive predominantly to β isoform, but crossreactive to other isoforms to a lesser extent	0.4	0.04
β	IBL	18641	N-terminal	Rabbit	Not crossreactive to other isoforms	2	1
γ	IBL	18647	C-terminal	Rabbit	Not crossreactive to other isoforms	5	0.2
ε	IBL	18643	C-terminal	Rabbit	Not crossreactive to other isoforms	2	1
ζ	IBL	18644	N-terminal	Rabbit	Not crossreactive to other isoforms	2	0.5
η	IBL	18645	N-terminal	Rabbit	Not crossreactive to other isoforms	5	1
θ (τ)	SC	sc-732	C-terminal	Rabbit	Not crossreactive to other isoforms	0.4	0.04
θ (τ)	IBL	10017	Recombinant, whole	Mouse	Minimally crossreactive to σ isoform	1	1
σ	IBL	18642	C-terminal	Rabbit	Not crossreactive to other isoforms	1	1

Abbreviations: SC, Santa Cruz Biotechnology; IBL, Immunobiological Laboratory. The specificity of the antibodies (IBL) is also shown on Supplementary Figure 1 at *http://www.amjpathol.org*.

Journal of Pathology website (*http://www.amjpathol.org*). The tissues were fixed with 4% paraformaldehyde (PFA) or 10% neutral formalin and embedded in paraffin. For the controls, tissue sections were prepared from the autopsied brains of six non-MS neurological and psychiatric disease cases that include a 47-year-old man with acute cerebral infarction who died of sepsis (no. 719), an 84-year-old man with acute cerebral infarction who died of disseminated intravascular coagulation (no. 786), a 62-year-old man with old cerebral infarction who died of pancreatic cancer (no. 789), a 56-year-old man with old cerebral infarction who died of myocardial infarction (no. 807), a 36-year-old woman with schizophrenia who died of lung tuberculosis (no. 523), and a 61-year-old man with schizophrenia who died of asphyxia (no. 826). In addition, they were prepared from the autopsied brains of six neurologically normal patients that include a 79-year-old woman who died of hepatic cancer (no. G6), a 75-year-old woman who died of breast cancer (no. G7), a 60-year-old woman who died of external auditory canal cancer (no. G8), a 74-year-old woman who died of gastric and hepatic cancers (no. G9), an 83-year-old woman who died of gastric cancer and myocardial infarction (no. A2623), and a 65-year-old man who died of liver cirrhosis and bronchopneumonia (no. A2647). Autopsies on all patients were performed at the National Center Hospital for Mental, Nervous, and Muscular Disorders, NCNP, Tokyo, Japan. Written informed consent was obtained in all cases.

Immunohistochemistry and Immunocytochemistry

After deparaffination, the tissue sections were heated by microwave at 95°C for 10 minutes in 10 mmol/L citrate sodium buffer (pH 6.0). They were then treated at room temperature for 15 minutes with 3% H_2O_2-containing methanol. For vimentin immunolabeling, the tissue sections were pretreated with 0.125% trypsin solution (Nichirei, Tokyo, Japan) at 37°C for 15 minutes. They were then incubated with 10% normal goat serum containing phosphate-buffered saline (PBS) at room temperature for 15 minutes to block nonspecific staining. The sections were incubated in a moist chamber at 4°C overnight with a panel of 14-3-3 isoform-specific antibodies or with antibodies broadly reactive against all isoforms listed in Table 1. The antibodies were obtained from Immunobiological Laboratory (IBL), Gumma, Japan, and Santa Cruz Biotechnology, Santa Cruz, CA. The specificity of the antibodies from IBL is shown in Supplementary Figure 1 on The American Journal of Pathology website, and additional information on those of Santa Cruz Biotechnology is available on the supplier's website (*www.scbt.com*). After washing with PBS, the tissue sections were labeled at room temperature for 30 minutes with peroxidase-conjugated secondary antibodies (Simple Stain MAX-PO kit, Nichirei) followed by incubation with a colorizing solution containing diaminobenzidine tetrahydrochloride and a counterstain with hematoxylin. To identify cell types expressing the 14-3-3 protein, adjacent sections were stained with the following antibodies: rabbit polyclonal antibody against GFAP (N1506; DAKO, Carpinteria, CA), rabbit polyclonal antibody against vimentin (H-84; Santa Cruz Biotechnology), mouse monoclonal antibody against vimentin (V9; Santa Cruz Biotechnology), rabbit polyclonal antibody against myelin basic protein (N1546; DAKO), mouse monoclonal antibody against CD68 (N1577; DAKO), and mouse monoclonal antibody against 70-kd and 200-kd neurofilament proteins (2F11; Nichirei). For negative controls, sections were incubated with a rabbit-negative control reagent (DAKO) instead of primary antibodies. The optimum concentrations of these antibodies and incubation periods were determined according to the supplier's instruction.

For double-labeling immunocytochemistry, cells on cover glasses were fixed with 4% PFA in 0.1 mol/L phos-

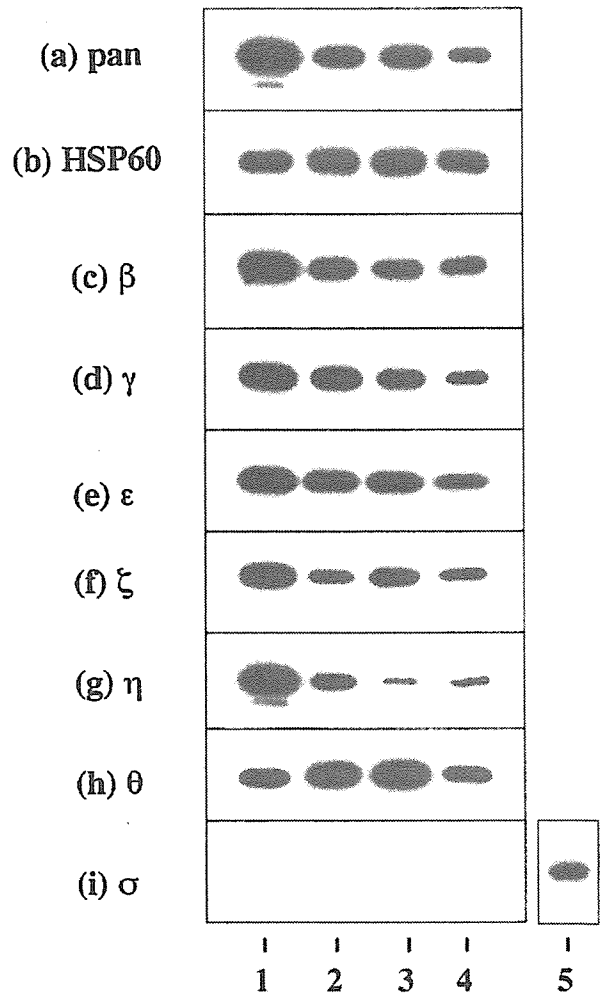

Figure 1. Constitutive expression of 14-3-3 isoforms in cultured human cells. Two μg of total protein extract isolated from brain tissues or cultured cells incubated in 10% FBS-containing medium were processed for Western blot analysis using a battery of 14-3-3 isoform-specific antibodies or the antibodies broadly reactive against all of the isoforms listed in Table 1, or the antibody against the house-keeping gene product HSP60. **a** to **i** indicate the following antibody specificity: **a**, all isoforms; **b**, HSP60; **c**, β; **d**, γ; **e**, ε; **f**, ζ; **g**, η; **h**, θ; and **i**, σ. **Lanes 1** to **4** represent homogenate of the human cerebrum (**lane 1**), NTera2-derived differentiated neurons (NTera2-N) (**lane 2**), U-373MG astrocytoma cells (**lane 3**), fetal human astrocytes (AS1F™) (**lane 4**), and HeLa cervical carcinoma cells (**lane 5**).

phate buffer (pH 7.4) at room temperature for 10 minutes, followed by incubation with PBS containing 0.5% Triton X-100 at room temperature for 20 minutes.[26] The cells were then incubated at room temperature for 30 minutes with a mixture of 14-3-3 isoform-specific antibody and rat monoclonal anti-GFAP antibody (2.2B10) or V9 antibody. Next, they were incubated at room temperature for 30 minutes with a mixture of rhodamine-conjugated anti-rabbit IgG and fluorescein isothiocyanate-conjugated anti-rat or mouse IgG (ICN-Cappel, Aurora, OH). After several washes, cover glasses were mounted on the slides with glycerol-polyvinyl alcohol, and the slides were examined under a Nikon ECLIPSE E800 universal microscope equipped with fluorescein and rhodamine optics. Negative controls were processed following these steps except for exposure to primary antibody.

Cell Culture

Two different sources of cultured human astrocytes were used. One was fetal human astrocytes named AS1477, provided by Drs. K. Watabe and S. U. Kim of the University of British Columbia, Vancouver, BC, Canada. They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (feeding medium). The other was astrocytes named AS-BW, whose differentiation was induced from neuronal progenitor (NP) cells. NP cells isolated from the brain of a human fetus at 18.5 weeks of gestation were obtained from BioWhittaker (Walkersville, MD). NP cells plated on a polyethyleneimine-coated surface were incubated in DMEM/F-12 medium containing an insulin-transferrin-selenium supplement (Invitrogen, Carlsbad, CA), 20 ng/ml recombinant human epidermal growth factor (Higeta, Tokyo, Japan), 20 ng/ml recombinant human basic fibroblast growth factor (PeproTech EC, London, UK), and 10 ng/ml recombinant human leukemia inhibitory factor (Chemicon, Temecula, CA) (NP medium).[35] For the induction of astrocyte differentiation, NP cells were incubated for several weeks in feeding medium instead of NP medium. This incubation induced vigorous proliferation and differentiation of astrocytes accompanied by a rapid reduction in nonastroglial cell types. Newborn mouse astrocytes were prepared as previously described.[26] In some experiments, cultured human and mouse astrocytes were plated at subconfluent density and incubated for 7 days in serum-free DMEM/F-12 medium supplemented with insulin-transferrin-selenium without inclusion of any other growth factors or in 10% FBS-containing DMEM/F-12 medium supplemented with insulin-transferrin-selenium.

Human cell lines such as U-373MG astrocytoma, NTera2 teratocarcinoma and HeLa cervical carcinoma were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and the American Type Culture Collection (Rockville, MD). For the induction of neuronal differentiation, NTera2 cells maintained in the undifferentiated state (NTera2-U) were incubated for 4 weeks in feeding me-

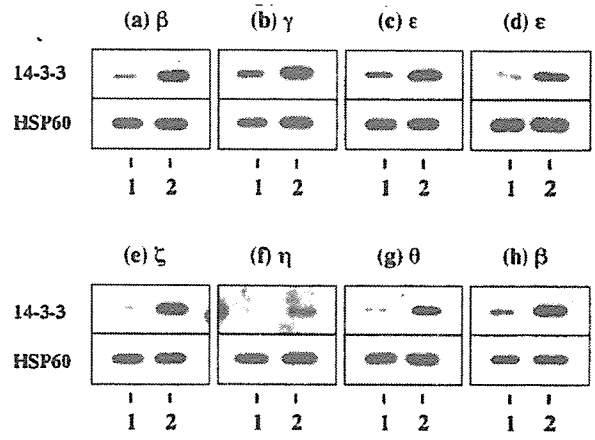

Figure 2. Growth-dependent expression of various 14-3-3 isoforms in cultured human astrocytes. Human and mouse astrocytes were plated at subconfluent density and incubated for 7 days in the serum-free culture medium or in 10% FBS-containing culture medium. Two μg of total protein extract was processed for Western blot analysis using a battery of 14-3-3 isoform-specific antibodies or with the antibodies broadly reactive against all isoforms (**top**). After stripping the antibodies, identical blots were relabeled with the antibody against HSP60 for the standardization of expression levels (**bottom**). **a** to **g** (**top**) indicate the expression of β (**a**), γ (**b**), ε (**c**), ζ (**e**), η (**f**), and θ (**g**) in human astrocytes (AS1477); ε in human astrocytes (AS-BW) (**d**); and β in mouse astrocytes (**h**). **Lanes 1** and **2** represent the cells cultured under the serum-free growth-arrested condition (**lane 1**) or the serum-containing growth-promoting condition (**lane 2**). Additional data are shown in supplementary Figure 2 on the American Journal of Pathology website.

Table 2. Differential Expression of Seven 14-3-3 Isoforms in Glial Cells and Neurons in MS and Control Brains

Brains 14-3-3 isoforms/cell types	Astrocytes			Microglia/macrophages			Oligodendrocytes			Neurons		
	MS	OND	NNC	MS	OND	NNC	MS	OND	NNC	MS	OND	NNC
β	maj (++)	maj (−)	no (−)	maj (++)	maj (+−)	no (−)	min (+)	no (−)	no (−)	maj (++)	maj (++)	maj (++)
γ	min (++)	min (−)	no (−)	min (++)	min (−)	no (−)	no (−)	no (−)	no (−)	maj (++)	maj (++)	maj (++)
ε	maj (++)	maj (−+)	min (+)	no (−)	no (−)	no (−)	no (−)	no (−)	no (−)	min (+)	min (+)	min (+)
ζ	maj (++)	maj (−+)	no (−)	maj (++)	maj (+−)	no (−)	no (−)	no (−)	no (−)	maj (++)	maj (++)	maj (++)
η	maj (++)	maj (−+)	no (−)	maj (++)	min (+−)	no (−)	no (−)	no (−)	no (−)	maj (++)	maj (++)	maj (++)
θ	min (+)	min (−)	no (−)	no (−)	no (−)	no (−)	min (++)	min (++)	min (+)	min (+)	min (+)	min (+)
σ	min (++)	min (−+)	min (+)	no (−)	no (−)	no (−)	no (−)	no (−)	no (−)	no (−)	no (−)	no (−)

The present study includes four MS cases numbered #791, 744, 609, and 544 whose clinical profiles are given in a supplementary table on the AJP website, six non-MS neurological and psychiatric disease cases (OND) composed of #719 acute cerebral infarction, #786 acute cerebral infarction, #789 old cerebral infarction, #807 old cerebral infarction, #523 schizophrenia, and #626 schizophrenia, and six neurologically normal cases (NNC) composed of #G6, #G7, #G8, #G9, #A2623, and #A2647, whose profiles are described in the Materials and Methods section.

The population size of the immunoreactive cells is expressed as maj, major (large) population; min, minor (small) population; and no, almost no population. The intensity of immunoreactivity is graded as (−) negative, (+) weak, and (++) intense.

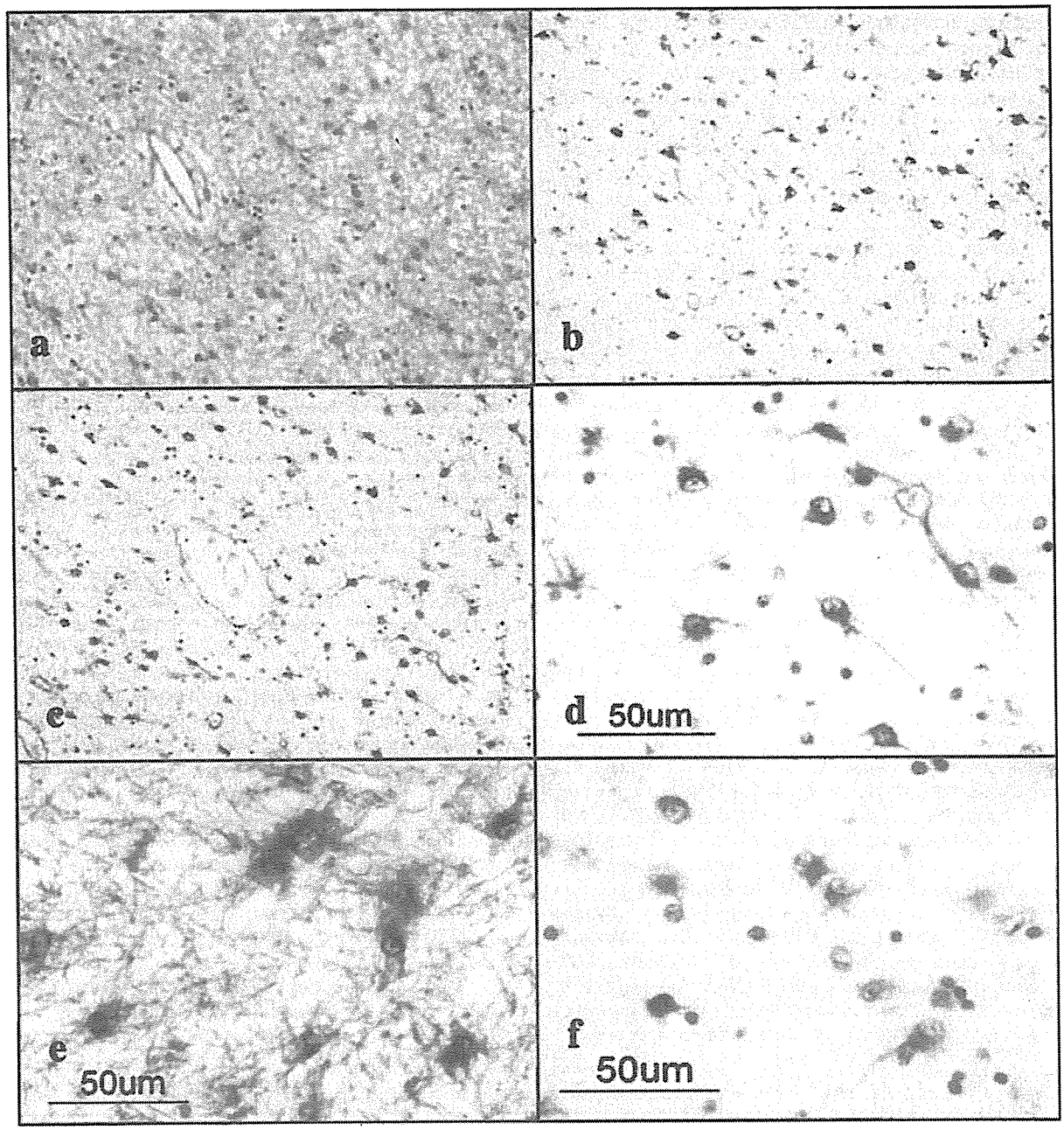

Figure 3. The 14-3-3 ε isoform is expressed in reactive astrocytes in chronic demyelinating lesions of MS. MS brain tissues were processed for immunohistochemical analysis using ε isoform-specific antibody or the antibody against GFAP or vimentin. **a** to **f** represent the following: **a:** no. 711 MS, chronic active demyelinating lesions in the subcortical white matter of the frontal lobe (H&E). **b:** No. 711 MS, the area corresponding to **a** (GFAP). Many reactive astrocytes are stained. **c:** No. 711 MS, the area corresponding to **a** (ε). Many reactive astrocytes are stained. **d:** No. 711 MS, a higher magnification view of **c** (ε). Reactive astrocytes are stained. **e:** No. 511 MS, chronic inactive demyelinating lesions in the optic nerve (ε). Reactive astrocytes and the glial scar are stained. **f:** No. 711 MS, chronic active demyelinating lesions in the subcortical white matter of the frontal lobe (vimentin). Reactive astrocytes are stained.

dium containing 10^{-5} mol/L *all trans* retinoic acid (Sigma, St. Louis, MO), replated twice and then plated on a surface coated with Matrigel Basement Membrane Matrix (Becton Dickinson, Bedford, MA). They were incubated for another 2 weeks in feeding medium containing a cocktail of mitotic inhibitors, resulting in the enrichment of differentiated neurons (NTera2-N).[36]

Western Blot Analysis

To prepare total protein extract for Western blot analysis, the cells and tissues were homogenized in RIPA lysis buffer composed of 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and a cock-

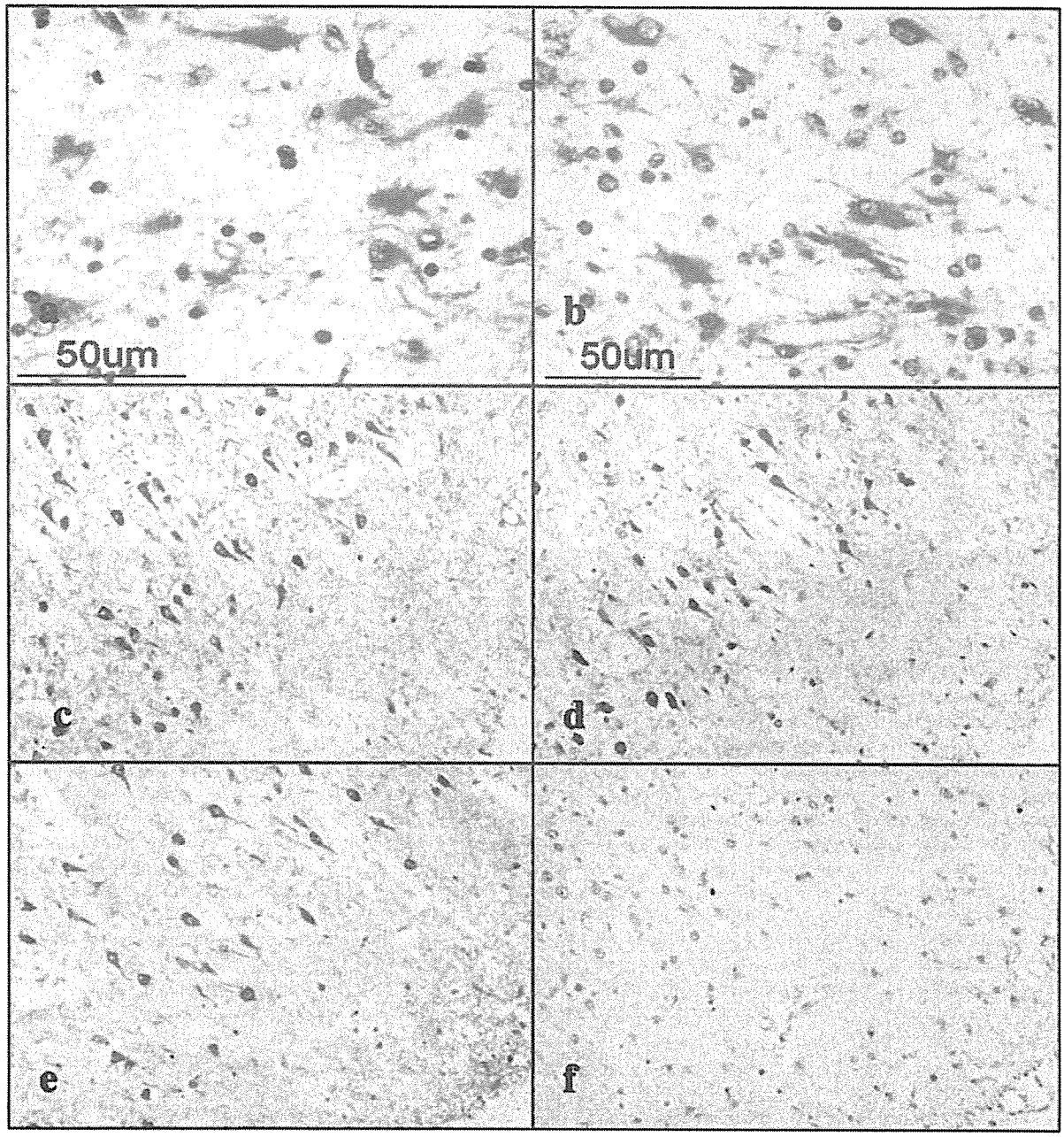

Figure 4. Expression of various 14-3-3 isoforms in reactive astrocytes and cortical neurons in MS brain. MS brain tissues were processed for immunohistochemical analysis using a battery of 14-3-3 isoform-specific antibodies. **a** to **f** represent the following: **a:** no. 711 MS, chronic active demyelinating lesions in the subcortical white matter of the frontal lobe (β). Reactive astrocytes are stained. **b:** No. 711 MS, chronic active demyelinating lesions in the subcortical white matter of the frontal lobe (ζ). Reactive astrocytes are stained. **c:** No. 711 MS, the cerebral cortex of the frontal lobe (γ). Cortical neurons are stained. **d:** No. 711 MS, the area corresponding to **c** (η). Cortical neurons are stained. **e:** No. 711 MS, the area corresponding to **c** (ζ). Cortical neurons are stained. **f:** No. 711 MS, the area corresponding to **c** (ε). Cortical neurons are devoid of staining.

tail of protease inhibitors (Roche Diagnositics, Mannheim, Germany), followed by centrifugation at 12,000 rpm at room temperature for 20 minutes. The supernatant was collected for separation on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel and the protein concentration was determined by a Bradford assay kit (Bio-Rad, Hercules, CA). After gel electrophoresis, the protein was transferred onto nitrocellulose membranes and immunolabeled at room temperature overnight with a panel of anti-14-3-3 protein antibodies listed in Table 1. Then, the membranes were incubated at room temperature for 30 minutes with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology). The specific reaction was visualized with a Western blot detection system using a chemiluminescent substrate (Pierce, Rockford, IL). After the antibodies were stripped