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Detection of human T-lymphotropic virus type I $p40^{tax}$ protein in cerebrospinal fluid cells from patients with human T-lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis

Takashi Moritoyo*[,1,2], Shuji Izumo[2], Hiroyoko Moritoyo[1], Yuetsu Tanaka[5], Yoshimasa Kiyomatsu[6], Masahiro Nagai[2], Koichiro Usuku[3], Masaru Sorimachi[4] and Mitsuhiro Osame[1]

[1]Third Department of Internal Medicine, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan; [2]Center for Chronic Viral Diseases, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan; [3]Medical Informatics, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan; [4]First Department of Physiology, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520; Japan; [5]Department of Biosciences, School of Science, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-0829, Japan; [6]Olympus, 3-4 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

We investigated the role of viral transcripts of human T-lymphotropic virus type I (HTLV-I) in the cerebrospinal fluid (CSF) cells and peripheral blood mononuclear cells (PBMCs) of patients with human T-lymphotropic virus type I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP). To detect the HTLV-I $p40^{tax}$ protein, we developed a new sensitive method of immunohistochemistry combined with tyramide signal amplification and quantitative analysis. Seven patients with HAM/TSP were examined. As controls, four patients with other neurological diseases were examined; two of these patients were infected with HTLV-I and the other two were not. Both the CSF cells and PBMCs were reacted with a monoclonal antibody, Lt-4, for $p40^{tax}$ protein, followed by secondary antibody labeled with horseradish peroxidase. This was visualized by fluorescein directly labeled with tyramide and the number of positive cells was quantified with a Laser Scanning Cytometer. In the samples from patients with HAM/TSP, the HTLV-I $p40^{tax}$ protein was successfully detected by tyramide signal amplification, but not without it. In HAM/TSP patients, 0.04–1.16% of the CSF cells and 0.02–0.54% of PBMCs were positive for the HTLV-I $p40^{tax}$ protein, respectively. The expression of the HTLV-I $p40^{tax}$ protein in the CSF cells was more frequent than that in PBMCs in both HAM/TSP patients and HTLV-I carriers, and was also more frequent in the patients with HAM/TSP of shorter duration of illness. This technique could be a powerful tool to investigate the pathogenic mechanism of diseases associated with HTLV-I.

Keywords: tyramide signal amplification; laser scanning cytometer; HTLV-I; HAM; TSP

Introduction

Human T-lymphotropic virus type I (HTLV-I) is associated with several diseases, including adult T-cell leukemia (ATL) and human T-lymphotropic virus type I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP). In a study using *in situ* hybridization, we previously reported that the HTLV-I tax gene, which is known to induce variable immune reactions, is expressed in $CD4^+$ T lymphocytes in areas of inflammation and white matter destruction in the spinal cord lesions of HAM/TSP (Moritoyo *et al*, 1996). The amount of the product of HTLV-I tax gene expression (HTLV-I $p40^{tax}$) *in vivo* is known to be too low to detect by conventional methods, which makes difficult a full understanding of the pathogenic mechanism of the viral transcription in patients with HAM/TSP. To detect the HTLV-I $p40^{tax}$ protein, we developed a new sensitive method of immunohistochemistry com-

Correspondence: T Moritoyo
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bined with tyramide signal amplification and quantitative analysis with Laser Scanning Cytometer (LSC). By this experimental approach, we unambiguously detected HTLV-I p40^{tax} protein in cerebrospinal fluid (CSF) cells and peripheral blood mononuclear cells (PBMCs) of patients with HAM/TSP, and describe in this article the role of viral transcripts of HTLV-I.

Results

Specificity of this detection system

The specificity of this detection system using Tyramide Signal Amplification (TSA) (NEN™, Boston, MA, USA) was documented in the HTLV-I-producing T-cell line (MT-2 cells) and virus-negative T-cell line (CEM cells). Primary antibody

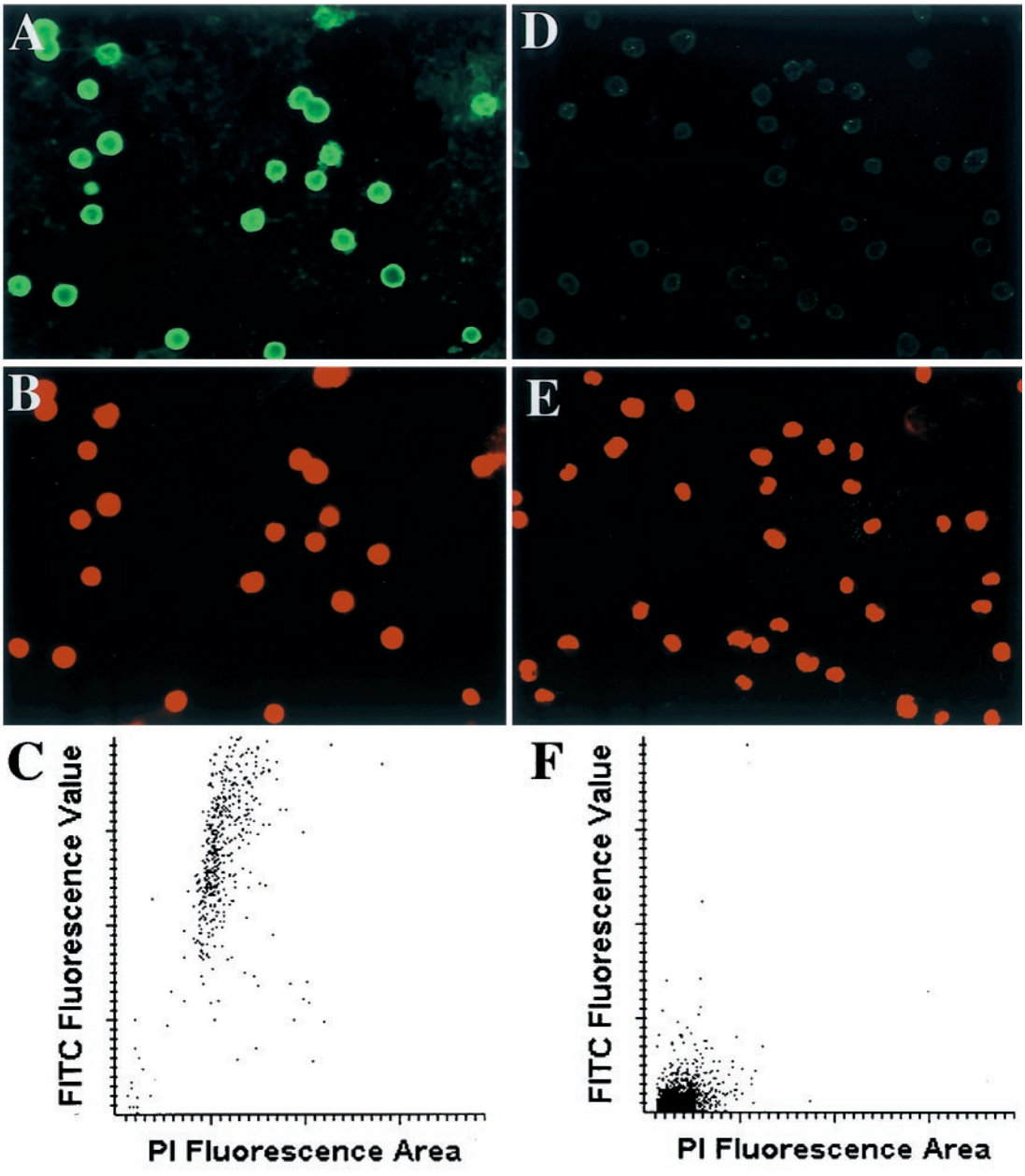

Figure 1 Detection of the HTLV-I p40^{tax} protein with TSA in MT-2 cells (**A**–**C**) and CEM cells (**D**–**F**). On its display, the LSC plotted the FITC fluorescence value (total fluorescence value of fluorescein of an individual cell, range 0–200) *vs* the PI fluorescence area (the area size of an individual nucleus calculated by the staining with propidium iodide for each cell). Most MT-2 cells exhibited a high value of fluorescence (mean 141.2, CV% 23.0) and CEM cells showed the background level of staining with Lt-4 antibody (mean 6.2, CV% 94.4). The cells on the display were recalled on the monitor of LSC and observed through the LSC microscope with both NIBA filter for fluorescein and WIG filter for propidium iodide (Olympus, Tokyo, Japan) in the same field. MT-2 cells were strongly stained with HTLV-I p40^{tax} antibody (Lt-4, green) (**A**), and their nuclei were stained with propidium iodide (red) (**B**). CEM cells were only stained with propidium iodide (red) (**D** and **E**). (**A**, **B**, **C** and **D**: Magnification ×200).

for the HTLV-I p40^{tax} protein (Lt-4) (Lee *et al*, 1989) strongly stained MT-2 cells green (Figure 1A). Propidium iodide counterstained nuclei red in those regions where HTLV-I p40^{tax} protein was also localized (Figure 1B). In CEM cells, no positive staining was observed (Figure 1D), but only red nuclear staining with propidium iodide (Figure 1E). Figure 1D and F showed the level of background staining with Lt-4. These results were consistent with those visualized using diaminobenzidine (DAB) as a substrate for horseradish peroxidase (HRP). Other controls, including elimination of the first antibody or replacement of the first antibody with normal mouse immunoglobulin (IgG3), anti-HIV p24 antibody (Dako, Carpinteria, CA, USA) or anti-chymotrypsin antibody (Chemicon, Temecula, CA, USA) did not result in detectable staining of cells. The background levels of staining with these controls were equivalent to the level of CEM cells stained with the Lt-4 antibody.

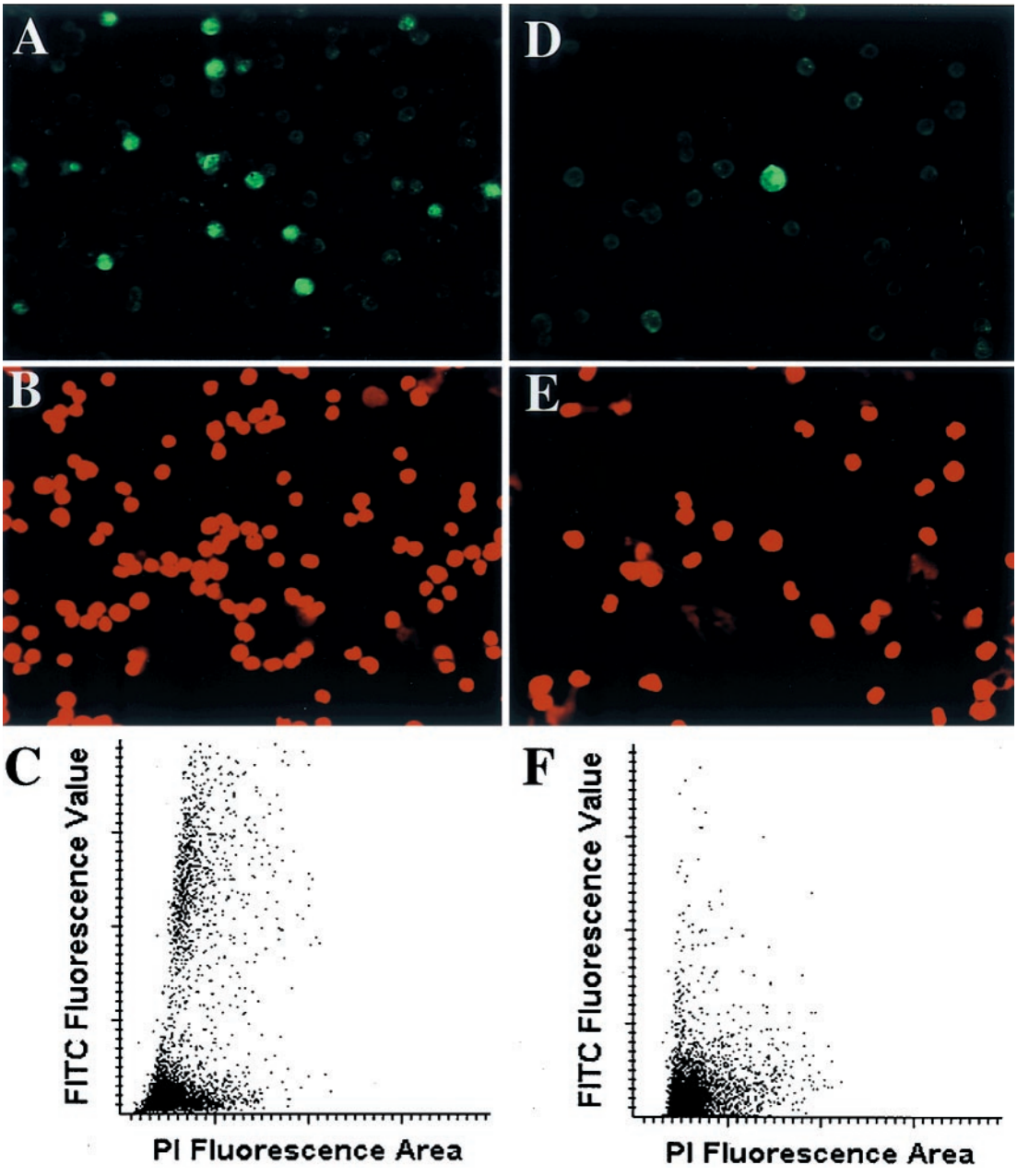

Figure 2 Experiments using artificial cell mixtures of MT-2 cells and CEM cells. The cell mixture contained 10% (**A**–**C**), 1% (**D**–**E**) and 0.01% (not shown) of MT-2 cells. For estimating the accuracy and the experimental variation of this detection system, we triplicated the slides of each cell mixture, counted the percentage of positive cells and calculated the mean and the percentage of coefficient of variance. (**A** and **D**: NIBA filter; **B** and **E**: WIG filter; **A** and **B**: Magnification ×100; **D** and **E**: Magnification ×200).

The cells on the slides were scanned with a Laser Scanning Cytometer (LSC) (Olympus, Tokyo, Japan) using the argon ion laser that recognized cells with red fluorescence stained with propidium iodide. At the same time, the values of fluorescence of individual cells were quantified with LSC and plotted on the display, where the total number of cells was automatically counted (Figure 1C and F). The total number of positive cells were manually counted, as all the individual cells on the display were recalled and observed using the LSC microscope, and in this manner the real positive staining of each individual cell was confirmed and the artifacts eliminated.

Experiments using artificial cell mixture of MT-2 cells and CEM cells estimated the accuracy and the experimental variation of this detection assay at the lower concentration of positive cells such as 10% (Figure 2A–C), 1% (Figure 2D–F) and 0.01% (not shown). We triplicated the slides of each cell mixture, counted the percentage of positive cells and calculated the mean and the percentage of coefficient of variance (CV%). The mean percentage, CV% was 9.25, 4.1 for 10% positive cells, 0.92, 9.3 for 1% and 0.092, 18.3 for 0.01%, respectively.

HTLV-1 p40^{tax} protein expression in CSF cells and PBMCs

CSF cells and PBMCs from seven patients with HAM/TSP were examined. HAM/TSP was diagnosed according to the criteria outlined by the World Health Organization (WHO, 1989). As controls, the cells from four patients with other neurological disorders were examined; two of these patients were infected with HTLV-I (HTLV-I carrier) and two were not (Table 1). In the samples from patients with HAM/TSP and in those from HTLV-I carriers, HTLV-I p40^{tax} protein was successfully detected with TSA (Figure 3A–F). However it was difficult to discriminate the positive signals because the color intensity of cells was faint when DAB was used as the substrate for HRP. The same controls for MT-2 cells and CEM cells as described in the former section were also used to avoid the false-positive signals, and the background staining levels of those controls were equivalent to that of CEM cells stained with Lt-4 antibody. The total numbers of cells and positive cells were counted using LSC, and the ratio of positive cells was calculated once, because the number of samples was limited and all the slides were used for many controls to eliminate the false-positive signals. In HAM/TSP patients, 0.04–1.16% of CSF cells and 0.02–0.54% of PBMCs were positive for HTLV-I p40^{tax} protein, respectively. In HTLV-I carriers 0.26–0.29% of CSF cells and 0.02–0.13% of PBMCs were positive. No positive signals were revealed in either the CSF cells or PBMCs from HTLV-I-negative patients. Expression of HTLV-I p40^{tax} protein was more frequent in CSF cells than in PBMCs in both HAM/TSP patients and HTLV-I carriers ($P<0.01$) (Figure 4). The expression of the HTLV-I p40^{tax} protein in CSF cells was also more frequent in patients with HAM/TSP of shorter duration of illness than in those with longer duration of illness ($P<0.05$) (Figure 5). There was no relationship between HTLV-I p40^{tax} expression and grade of motor disability, titers of anti-HTLV-I antibody in serum or CSF, or values of neopterin in CSF.

Discussion

In HAM/TSP patients, antibodies against the p40^{tax} gene product of HTLV-I are frequently positive (Kamihira *et al*, 1989), and the virus-specific immune response directed to the HTLV-I tax gene product is predominantly observed (Jacobson *et al*, 1990, 1992; Kannagi *et al*, 1991, 1992; Bangham *et*

Table 1 Clinical profiles of patients

Patient No.	*Age (year)*	*Sex*	*Diagnosis*	*Duration of Illness*	*Motor Disability Score*	*Anti-HTLV-I Ab (PA) in Serum*	*Anti-HTLV-I Ab (PA) in CSF*	*Nepterin in CSF (pmol/ml)*
1	49	M	HAM/TSP	1 year, 4 months	6	×2048	×4096	136.4
2	67	M	HAM/TSP	1 year, 5 months	9	×8192	×131072<	187.8
3	72	F	HAM/TSP	2 years	4	×16384	×512	62.1
4	44	F	HAM/TSP	5 years, 2 months	6	×2048	×1024	186.7
5	42	F	HAM/TSP	8 years	4	×8192	×16	99.7
6	42	F	HAM/TSP	10 years	4	×4096	×128	108
7	32	M	HAM/TSP	12 years	4	×512	×512	129.5
8	48	M	HMSN			×1024	×8	19.8
9	72	F	Transverse myelitis			×1024	×1024	23.2
10	70	M	Crow-Fukase syndrome			Negative	Negative	54.9
11	42	F	Myelopathy			Negative	Negative	17.9

HMSN = Hereditary motor and sensory neuropathy; Ab = antibody; PA = particle agglutination method; CSF = cerebrospinal fluid; For Motor Disability Score see Nakagawa *et al*, 1995

al, 1996). However, conventional immunohistochemical methods are not sensitive enough to detect the viral protein of HTLV-I in freshly prepared samples from patients with HAM/TSP, although HTLV-I antigen expression has been detected in cultured peripheral blood mononuclear cells (Minato *et al*, 1988). We therefore undertook an investigation of the protein of the viral gene transcription underlying the pathogenic mechanisms of HAM/TSP using a newly developed and more sensitive method of immunohistochemistry.

Recent development of Tyramide Signal Amplification (TSA) (Bobrow *et al*, 1989) has greatly improved the detection ability of immunohistochemistry. Tyramide is a phenolic compound that reacts with electron rich moieties on the surface like

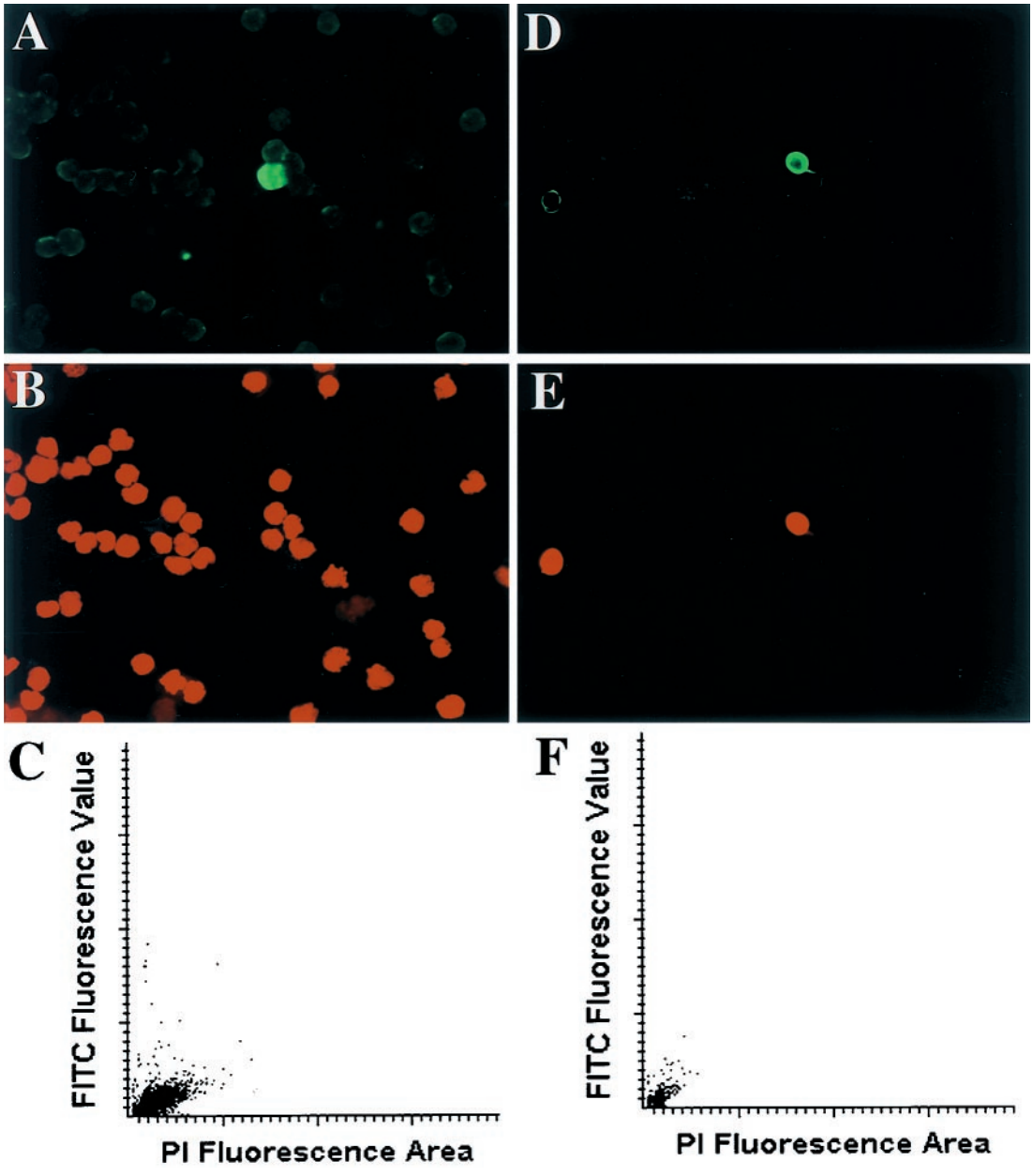

Figure 3 Detection of the HTLV-I p40^{tax} protein with TSA in the samples from patients with HAM/TSP. HTLV-I p40^{tax} protein (green) and nuclear staining of propidium iodide (red) were observed through the LSC microscope in PBMCs (**A** and **B**) and in CSF cells (**D** and **E**). Positive cells of these samples exhibited relatively higher fluorescence values among the cells appeared on the graph and clearly discriminated from the background staining and artifacts when we observed these cells with the LSC microscope. But those positive cells showed lower fluorescence values (less than 90) than MT-2 cells (**C** and **F**). The total number of positive cells was manually counted using the LSC microscope after all the cells on the graph were recalled (**A** and **D**: NIBA filter; **B** and **E**: WIG filter; **A**, **B**, **D** and **E**: Magnification $\times 400$).

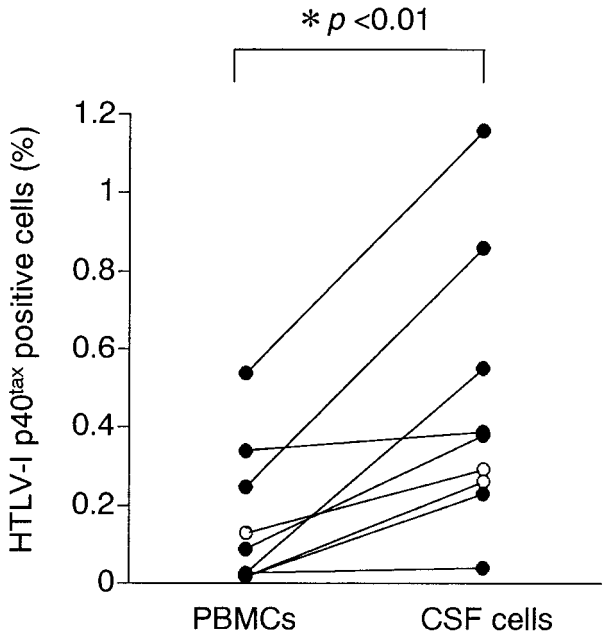

Figure 4 HTLV-I p40^{tax} expression in the CSF cells and PBMCs. The closed circles represent patients with HAM/TSP and the open circles represent HTLV-I carriers. The expression of the HTLV-I p40^{tax} protein was more frequent in the CSF cells than in PBMCs in both HAM/TSP patients and HTLV-I carriers ($P<0.01$ by two sample *t*-test).

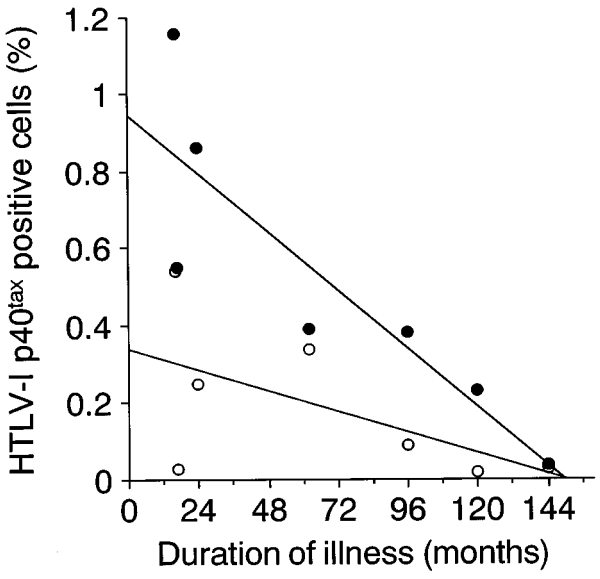

Figure 5 Relationship of HTLV-I p40^{tax} expression to duration of illness of patients with HAM/TSP. The expression of HTLV-I p40^{tax} in CSF cells (closed circles) was more frequent in patients with HAM/TSP of shorter duration of illness ($P<0.05$ by Spearman's correlation coefficient).

proteins in tissues. HRP catalyzes this reaction via free radical formation. Despite the improvement of the signal intensity, the background noise often results in false-positive signals when TSA is used with a biotin-streptavidin system. We therefore used Envision (Dako, Carpinteria, CA, USA), which has been labeled with many HRPs using dextran polymer, as a secondary antibody. This antibody is finally visualized with fluorescein labeled directly with tyramide. Using this system, we could decrease the background without losing the positive signal intensity, resulting in a system of improved detection ability. The HTLV-I p40^{tax} antigen was successfully detected not only in the HTLV-I-producing T-cell line but also in the CSF cells and PBMCs of patients with HAM/TSP and in those of HTLV-I carriers with this system.

The Laser Scanning Cytometer (LSC) (Kamentsky and Kamentsky 1991) is a microscope-based scanning cytometer that generates data comparable to that by flow cytometry. But the LSC has certain advantages over the conventional flow cytometry. The LSC uses a microscopic slide on which small numbers of spotted cells, such as those in the CSF ($<10^4$), can be scanned. Here these scanned cells were recalled and observed both on the monitor and the LSC microscope. This recall function enabled us to count the extremely small number of positive cells and to eliminate the artifacts completely. The use of a slide also greatly simplifies the complicated procedures of TSA and *in situ* hybridization. LSC might be considered a powerful tool for investigating those neurological diseases in which the CSF contains important clues regarding pathogenesis.

With the sensitivity of the method we employed, we could document the expression of HTLV-I p40^{tax} protein in uncultured samples from patients with HAM/TSP and from HTLV-I carriers. The value of fluorescein in PBMCs of HAM/TSP patients and HTLV-I carriers was not as high as in MT-2 cells. This supported the findings reported by Furukawa and associates, who used reverse transcriptase-mediated polymerase chain reaction to demonstrate that the expression level per infected cell of HTLV-I tax mRNA in HAM/TSP was not different from that in asymptomatic carriers (Furukawa *et al*, 1995). Although the limited number of cases and samples were analyzed in this study, our clear demonstration of the frequent expression of HTLV-I p40^{tax} protein in CSF cells and the correlation between this expression and duration of illness were in good agreement with the results of the HTLV-I tax gene expression and pathological findings in the spinal cord of patients with HAM/TSP, in which HTLV-I tax gene expression was predominant in patients with HAM/TSP of shorter duration of illness (Moritoyo *et al*, 1996). These findings, together with the clinical course of HAM/TSP patients (Nakagawa *et al*, 1995), suggest a close association between the expression of HTLV-I tax

protein in the spinal cord and the disease activity of patients with HAM/TSP, and evoke an important strategy for early-stage treatment of patients with HAM/TSP.

Materials and methods

Cell samples

CSF cells were cytocentrifuged immediately after the lumbar puncture. PBMCs were separated by Ficoll/Hypaque density gradients from heparinized blood samples using methods previously described (Ijichi *et al*, 1989). Both CSF cells and isolated PBMCs were spotted onto silane-coated slides (Perkin Elmer, Foster City, CA, USA), fixed with 4% paraformaldehyde for 30 min, and stored at -80°C until use. The HTLV-I-producing T-cell line (MT-2 cells) and virus-negative T-cell line (CEM cells) were cultured in RPMI 1640 with 10% fetal calf serum, penicillin G (50 u/ml), and streptomycin (50 mg/ml). Cells were washed in phosphate buffer saline (PBS)-CMF and processed similarly.

Immunohistochemistry

Both the CSF cells and PBMCs were restored at ambient temperature and incubated with 3% hydrogen peroxide for 5 min. Primary antibodies (mouse monoclonal) for HTLV-I p40^{tax} (Lt-4) (IgG3) (Lee *et al*, 1989), human immunodeficiency virus (HIV), p24 (IgG1) (Dako, Carpinteria, CA, USA) and chymotrypsin (IgG3) (Chemicon, Temecula, CA, USA) were diluted at 1 : 500, 1 : 10 and 1 : 1000, respectively. After incubation with primary antibody and subsequent washing, the cells were blocked with Blocking Buffer (NEN™, Boston, MA, USA), followed by incubation with the Dako Envision peroxidase-labeled polymer reagent as secondary antibody. We used both DAB as substrate for HRP and fluorescein labeled with tyramide in TSA-Direct (NEN™, Boston, MA, USA), which was catalyzed by HRP and was deposited close to the enzyme. Numerous fluoresceins labeled with tyramide were finally deposited and fluorescent signals were formed. After counterstaining of the cell nuclei with propidium iodide, the cells on the slide were automatically scanned and counted by the LSC, which then displayed the scanning results on its monitor. All the cells on the display were recalled and the total number of positive cells was manually counted using the LSC microscope.

Acknowledgments

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