Increased Production of Inflammatory Cytokines in Cultured CD4⁺ Cells from Patients with HTLV-I-Associated Myelopathy

YOSHIHIRO NISHIURA, TATSUFUMI NAKAMURA, KATSUHIRO ICHINOSE, SUSUMU SHIRABE, AKIRA TSUJINO, HIROFUMI GOTO, TAKAFUMI FURUYA and SHIGENOBU NAGATAKI

The First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki 852

NISHIURA, Y., NAKAMURA, T., ICHINOSE, K., SHIRABE, S., TSUJINO, A., GOTO, H., FURUYA, T. and NAGATAKI, S. *Increased Production of Inflammatory Cytokines in Cultured CD4⁺ Cells from Patients with HTLV-I-Associated Myelopathy.* Tohoku J. Exp. Med., 1996, **179** (4), 227–233 — We investigated the production of inflammatory cytokines derived from cultured T cells of peripheral blood lymphocytes (PBL) in 14 patients with HTLV-I-associated myelopathy (HAM). The production of inflammatory cytokines, such as tumor necrosis factor- α , interferon- γ , and granulocyte-macrophage colony stimulating factor, was significantly increased in patients with HAM, compared to HTLV-I seronegative controls. On the contrary, interleukin-4 production in cultured T cells was detected in only two patients with HAM, and not detected in HTLV-I seronegative controls. These results suggest that the production of inflammatory cytokines derived from TH1 cell population was simultaneously exaggerated in HAM patients. Interestingly, accelerated production of these cytokines was derived from CD4⁺ cells, which are main target cells in HTLV-I infection. These findings suggest that an inflammatory state in the central nervous system might be related to the pathogenesis of HAM. — HTLV-I-associated myelopathy; tumor necrosis factor- α ; interferon- γ ; granulocyte-macrophage colony stimulating factor; CD4⁺ cell

The main histopathological findings of human T-lymphotropic virus type I (HTLV-I)-associated myelopathy (HAM) is chronic inflammation characterized by perivascular cuffing with lymphocytes and parenchymal lymphocytic infiltration (Akizuki et al. 1987). Although both CD4⁺ and CD8⁺ T cells are activated in peripheral blood lymphocytes (PBL) of patients with HAM (Itoyama et al. 1989), the role of activated T cells in the formation of the pathological lesions of HAM is unclear. Umehara et al. (1993) recently reported that CD4⁺ cells, CD8⁺ cells and macrophages infiltrated active-chronic inflammatory lesions in the spinal cords of HAM patients with a short duration of illness. However,

---

Received January 5, 1996; revision accepted for publication June 17, 1996.

Address for reprints: Dr. Yoshihiro Nishiura, The First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852, Japan.

in inactive lesions of HAM patients with long duration of illness, CD8⁺ cells predominated over CD4⁺ cells. We have shown that transcripts of inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon- γ (IFN- γ) and interleukin-1 α (IL-1 α), were simultaneously up-regulated in peripheral blood mononuclear cells (PBMC) of patients with HAM, compared to either HTLV-I seropositive carriers or seronegative controls (Watanabe et al. 1995). To further clarify the source of these cytokines, we measured the levels of TNF- α , IFN- γ , GM-CSF and interleukin-4 (IL-4) in supernatants of cultured T cells and of CD4⁺ and CD8⁺ enriched populations from patients with HAM.

SUBJECTS AND METHODS

Fourteen patients with HAM (1 man and 13 women; mean age, 58.5 years; age range, 34 to 73 years) were studied. The diagnosis of HAM was based on the criteria described by WHO meeting in Kagoshima (Osame 1990). Controls consisted of 12 HTLV-I-seronegative individuals (5 men and 7 women; mean age, 54.4 years; age range, 32 to 77 years); 4 healthy individuals and patients with other neurological disorders, such as 3 spinocerebellar degeneration patients, 3 amyotrophic lateral sclerosis patients, 2 brain tumor patients were studied as the control.

Separation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood samples by Ficoll-Conray density-gradient centrifugation as described previously (Nishiura et al. 1994). Macrophages were removed by allowing the separated cells to adhere to the surfaces of Petri dishes (Becton Dickinson Co., Lincoln Park, NJ, USA). Further to remove B cells, the macrophage-depleted PBMC were incubated in Petri dishes coated with affinity-purified mouse anti-human immunoglobulin antibody (Organon Teknica Corp., West Chester, PA, USA) for 1 hr at 4°C. T cell-enriched population was prepared by gently removing the nonadherent cells, after which the cells were used as T cell enriched population. Thereafter they were incubated with mouse monoclonal antibody to CD4 or CD8 antigen (Immunotech S.A., Marseilles, France) for 30 min at 4°C, then incubated in Petri dishes coated with affinity-purified goat anti-mouse immunoglobulin antibody (Organon Teknica Corp.) for 1 hr at 4°C. The adherent CD4⁺ or CD8⁺ cells harvested by scraping (Sumitomo Bakelite Co., Tokyo) were washed with phosphate-buffered saline (PBS) and were incubated for 24 hr at 37°C in 5% CO₂. Then, they were used as CD4⁺ enriched population or CD8⁺ enriched population. To study inflammatory cytokines, each cell population (T cells, CD4 enriched, or CD8 enriched) was cultured at 1×10^6 cells/ml in RPMI 1640 supplemented with 20% fetal bovine serum (FBS; GIBCO, Grand Island, NY, USA) for 4 days after separated to each populations, then the culture medium was

centrifuged at 2500 rpm for 10 min and the supernatant was stored at -40°C until use. These CD4^{+} and CD8^{+} enriched populations were shown to have more than 95% cell specificity by a FACScan flow cytometer (Becton-Dickinson Immunocytometry System, San Jose, CA, USA).

Measurement of cytokines

$\text{TNF-}\alpha$, $\text{IFN-}\gamma$, GM-CSF and IL-4 levels were measured in the culture supernatants by using $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, GM-CSF and IL-4 EASIA (enzyme-amplified sensitivity immunoassay) Kit (Medgenix, Fleurus, Belgium). EASIA is based on an oligoclonal system in which several monoclonal antibodies are directed against distinct epitopes of each cytokine. The culture supernatant of 200 μl was needed for measurement of $\text{TNF-}\alpha$ and GM-CSF, 100 μl for IL-4, and 50 μl for $\text{IFN-}\gamma$. The culture supernatant and appropriate standards were added, in duplicate, to each cytokine monoclonal antibody-coated wells. This was followed by incubation with the anti-respective cytokine horseradish peroxidase conjugate at room temperature for 2 hr ($\text{TNF-}\alpha$, $\text{IFN-}\gamma$ and IL-4) or 4 hr (GM-CSF). After washing, the substrate solution (tetramethylbenzidine) was dispensed to each well and incubated for 15 min ($\text{IFN-}\gamma$ and GM-CSF) or 30 min ($\text{TNF-}\alpha$ and IL-4). The reaction was stopped with H_2SO_4 and the absorbances were measured using an optical densitometer. The minimum measurable level of each cytokine was 1.9 pg/ml for $\text{TNF-}\alpha$, 0.1 IU/ml for $\text{IFN-}\gamma$, 6.3 pg/ml for GM-CSF and 6.0 pg/ml for IL-4.

RESULTS

The levels of $\text{TNF-}\alpha$ ($p < 0.001$), $\text{IFN-}\gamma$ ($p < 0.001$) and GM-CSF ($p < 0.001$) were significantly increased in the culture supernatants of T cells derived from HAM patients, compared with HTLV-I-seronegative controls (Fig. 1). In contrast, IL-4 was detected in only two patients with HAM (level, 6.4 and 8.5 pg/ml) and not detected in all of HTLV-I-seronegative controls (data not shown).

To investigate which population of T cells produced $\text{TNF-}\alpha$, $\text{IFN-}\gamma$ and GM-CSF, we separated T cells to CD4^{+} and CD8^{+} enriched cells by the panning method and measured the levels of each cytokines in the culture supernatants. Culture supernatants of CD4^{+} enriched cells showed significantly higher levels of $\text{TNF-}\alpha$, $\text{IFN-}\gamma$ and GM-CSF compared with CD8^{+} enriched cells ($p < 0.05$, Fig. 2) in patients with HAM. There was no discernible relationship between the cytokines levels and the duration or severity of illness, the anti-HTLV-I antibody titers in serum and CSF and spontaneous PBL proliferation of HAM patients.

DISCUSSION

Recently we reported up-regulation of transcripts of inflammatory cytokines, including $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, GM-CSF and IL- 1α , in patients with HAM (Watanabe et al. 1995). In the present paper, we demonstrate significantly increased produc-

Fig. 1. TNF- α , IFN- γ and GM-CSF levels in culture supernatants of T cells derived from patients with HAM ($n=14$) and anti-HTLV-I seronegative controls ($n=12$). All cytokines levels in culture supernatants of T cells derived from patients with HAM were significantly higher than that of T cells from HTLV-I-seronegative controls. Differences were statistically significant by the Wilcoxon signed-rank test as follows: *** $p < 0.001$. Bar shows mean.

Fig. 2. TNF- α , IFN- γ and GM-CSF levels in culture supernatants of CD4⁺ and CD8⁺ T cells derived from patients with HAM ($n=8$). Culture supernatants of CD4⁺ cells derived from patients with HAM showed significantly higher levels of all cytokines compared to that of CD8⁺ cells. Differences were statistically significant by the Mann-Whitney U test as follows: * $p < 0.05$. Bar shows mean.

tion of TNF- α , IFN- γ and GM-CSF by T cells from patients with HAM. Since the replication of HTLV-I is increased in PBL of patients with HAM (Nagasato et al. 1991), it is conceivable that the tax gene product of HTLV-I activates the transcription of TNF- α , IFN- γ and GM-CSF. Interestingly, the increased production of inflammatory cytokines was mainly derived from CD4⁺ cells, which is the main population of target cells of HTLV-I, in patients with HAM. Therefore,

the high load of HTLV-I in patients with HAM might be related to the accelerated production of these inflammatory cytokines.

In mice, helper T cells can be separated into two distinct populations, TH1 and TH2, by differences in the pattern of cytokines synthesized (Mosmann and Coffman 1989). IFN- γ is derived from TH1 cells, and both TNF- α and GM-CSF are mainly produced by TH1 rather than TH2 cells. In contrast, IL-4 is derived from TH2 cells. In this report, IL-4 production in cultured T cells was undetectable in all but two patients with HAM in whom very low levels were found. TH1, TH2 lymphocytes are involved in distinct immune functions in humans (Del Prete et al. 1994). Our data are consistent with cytokine production by the activated TH1 cell population. IL-4, a pleiotropic cytokine for the immune system (Paul and Ohara 1987), suppresses IFN- γ and TNF- α production by T cells and macrophages. Conversely, IFN- γ suppresses IL-4 production by TH2 cells (Mosmann and Coffman 1989). Histological studies on CNS tissue obtained from HAM patients at autopsy indicated that an inflammatory process is involved in this disease. On the other hand, it was reported that IFN- γ and GM-CSF were increased in the cerebrospinal fluid (Kuroda et al. 1993; Kuroda and Matui 1993). The production of inflammatory cytokines derived from TH1 cells might be up-regulated because of decreased production of IL-4. Collectively, these findings may be relevant to the inflammatory status in the spinal cord of patients with HAM.

Although the precise role of the inflammatory cytokines in neuropathology of HAM is unclear, it is conceivable that these cytokines have pleiotropic functions against the cells which constitute the nervous system. For example, GM-CSF, a potent stimulator of macrophages, also induces the proliferation of microglia isolated from newborn mice (Suzumura et al. 1990). Recent studies also reveal elevated levels of TNF- α in the cerebrospinal fluid of patients infected with human immunodeficiency virus type 1 (Grimaldi et al. 1991), and intrathecal synthesis of TNF- α in patients with multiple sclerosis (Sharief et al. 1991). In this regard, Selmaj and Raine (1988) have reported that TNF- α causes demyelination and necrosis of oligodendrocytes in organotypic spinal cord cultures. TNF- α also causes morphological changes in the vascular endothelium (Sato et al. 1986). Moreover, IFN- γ induces class II major histocompatibility complex (MHC) antigens (Sztein et al. 1984) and may play an important role in eliciting a T cell-mediated immune response leading to CNS damage. T cell-mediated immune response and virus-induced, cytotoxic T cell-mediated demyelination might be important in the pathogenesis of HAM (Moore et al. 1989).

How HTLV-I infection causes chronic myelopathy is still unknown. However, recent reports, using polymerase chain reaction in situ hybridization, described that HTLV-I proviral DNA in the spinal cords lesions of HAM patients was only in infiltrated lymphocytes, not in any neuronal cells (Hara et al. 1994; Kashio et al. 1994). Therefore, increased levels of inflammatory cytokines,

produced by infiltrated CD4⁺ T cells in spinal cords of patients with HAM, may have a deleterious effects on the nervous system.

Acknowledgments

We thank Dr. Richard Yanagihara (LCNSS, NINDS, NIH, USA) for reviewing the manuscript. This work was supported in part by a grant from the Neuroimmunological Disease Research Committee of the Ministry of Health and Welfare of Japan.

References

- 1) Akizuki, S., Nakazato, O., Higuchi, Y., Tanabe, K., Setoguchi, M., Yoshida, S., Miyazaki, Y., Yamamoto, S., Sudou, S., Sannomiya, K. & Okajima, T. (1987) Necropsy findings in HTLV-I associated myelopathy. *Lancet*, **1**, 156-157.
- 2) Del Prete, G., Maggi, E. & Romagnani, S. (1994) Human Th1 and Th2 cells: Functional properties, mechanisms of regulation, and role in disease. *Lab. Invest.*, **70**, 299-306.
- 3) Grimaldi, L.M.E., Martino, G.V., Franciotta, D.M., Brustia, R., Castagna, A., Pristera, R. & Lazzarin, A. (1991) Elevated alpha-tumor necrosis factor levels in spinal fluid from HIV-1-infected patients with central nervous system involvement. *Ann. Neurol.*, **29**, 21-25.
- 4) Hara, H., Morita, M., Iwaka, T., Hatae, H., Itoyama, Y., Kitamoto, T., Akizuki, S., Goto, I. & Watanabe, T. (1994) Detection of human T-lymphotropic virus type I (HTLV-I) proviral DNA and analysis of T cell receptor V β CDR3 sequences in spinal cord lesions of HTLV-I-associated myelopathy/tropical spastic paraparesis. *J. Exp. Med.*, **180**, 831-839.
- 5) Itoyama, Y., Kira, J., Fujii, N., Goto, I. & Yamamoto, N. (1989) Increases in helper inducer T cells and activated T cells in HTLV-I-associated myelopathy. *Ann. Neurol.*, **26**, 257-262.
- 6) Kashio, N., Izumo, S., Ijichi, S., Hashimoto, K., Umehara, F., Higuchi, I. & Osame, M. (1994) Detection of HTLV-I provirus by in situ polymerase chain reaction in mononuclear cells infiltrating spinal cord lesions from HAM/TSP patients. *Brain Pathol.*, **4**, 498.
- 7) Kuroda, Y. & Matui, M. (1993) Cerebrospinal fluid interferon-gamma is increased in HTLV-I-associated myelopathy. *J. Neuroimmunol.*, **42**, 223-226.
- 8) Kuroda, Y., Matui, M., Takashima, H. & Kurohara, K. (1993) Granulocyte-macrophage colony-stimulating factor and interleukin-1 increase in cerebrospinal fluid, but not in serum, of HTLV-I-associated myelopathy. *J. Neuroimmunol.*, **45**, 133-136.
- 9) Moore, G.R.W., Traugott, U., Scheinberg, L.C. & Raine, C.S. (1989) Tropical spastic paraparesis: A model of virus-induced, cytotoxic T-cell-mediated demyelination? *Ann. Neurol.*, **26**, 523-530.
- 10) Mosmann, T.R. & Coffman, R.L. (1989) TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.*, **7**, 145-173.
- 11) Nagasato, K., Nakamura, T., Ohishi, K., Shibayama, K., Motomura, M., Ichinose, K., Tsujihata, M. & Nagataki, S. (1991) Active production of anti-human T-lymphotropic virus type I (HTLV-I) IgM antibody in HTLV-I-associated myelopathy. *J. Neuroimmunol.*, **32**, 105-109.
- 12) Nishiura, Y., Nakamura, T., Ichinose, K., Nagasato, K., Ohishi, K., Watanabe, H., Shirabe, S., Tsujihata, M. & Nagataki, S. (1994) Antiproliferative factor against the human glioblastoma cell line T98G identified in culture supernatants of CD4⁺ cells from patients with HTLV-I-associated myelopathy. *J. Neurol. Sci.*, **122**, 214-

- 219.
- 13) Osame, M. (1990) Review of WHO Kagoshima meeting and diagnostic guidelines for HAM/TSP. In: *Human Retrovirology: HTLV*, edited by W.A. Blattner, Raven Press, New York, pp. 191-197.
  - 14) Paul, W.E. & Ohara, J. (1987) B-cell stimulatory factor-1/interleukin 4. *Ann. Rev. Immunol.*, **5**, 429-459.
  - 15) Sato, N., Goto, T., Haranaka, K., Satomi, N., Nariuchi, H., Mano-Hirano, Y. & Sawasaki, Y. (1986) Actions of tumor necrosis factor on cultured vascular endothelial cells: Morphologic modulation, growth inhibition, and cytotoxicity. *J. Natl. Cancer Inst.*, **76**, 1113-1121.
  - 16) Selmaj, K.W. & Raine, C.S. (1988) Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann. Neurol.*, **23**, 339-346.
  - 17) Sharief, M.K., Phil, M. & Hentges, R. (1991) Association between tumor necrosis factor- α and disease progression in patients with multiple sclerosis. *N. Engl. J. Med.*, **325**, 467-472.
  - 18) Suzumura, A., Sawada, M., Yamamoto, H. & Marunouchi, T. (1990) Effects of colony stimulating factors on isolated microglia in vitro. *J. Neuroimmunol.*, **30**, 111-120.
  - 19) Sztein, M.B., Steeg, P.S., Johnson, H.M. & Oppenheim, J.J. (1984) Regulation of human peripheral blood monocyte DR antigen expression in vitro by lymphokines and recombinant interferons. *J. Clin. Invest.*, **73**, 556-565.
  - 20) Umehara, F., Izumo, S., Nakagawa, M., Ronquillo, A.T., Takahashi, K., Matsumuro, K., Sato, E. & Osame, M. (1993) Immunocytochemical analysis of the cellular infiltrate in the spinal cord lesions in HTLV-I-associated myelopathy. *J. Neuropathol. Exp. Neurol.*, **52**, 424-430.
  - 21) Watanabe, H., Nakamura, T., Nagasato, K., Shirabe, S., Ohishi, K., Ichinose, K., Nishiura, Y., Chiyoda, S., Tsujihata, M. & Nagataki, S. (1995) Exaggerated messenger RNA expression of inflammatory cytokines in human T-cell lymphotropic virus type I-associated myelopathy. *Arch. Neurol.*, **52**, 276-280.
-