in the sera of patients with AD. It is likely that when proteins levels are higher than average in sera and CSF in neurodegenerative diseases, there would be an increase in retrograde transport from the axon projections outside of central nervous system where the passage of plasma proteins is not as restricted as at the brain–blood barrier, or from axon terminini in the circumventricular region where the brain–blood barrier is not present. There is a possibility that an imbalance of cysteine proteases and their inhibitors and metabolic dysfunction in sera and CSF might occur in ALS cases, being related to the formation of Bunina bodies and accumulation of transferrin and cystatin C in Bunina bodies in the remaining anterior horn cells.

Transferrin, after binding two atoms of ferric iron, binds to the transferrin receptor on the cell membrane and is internalized along with the divalent metal transporter by receptor-mediated endocytosis. It is unknown how transferrin reaches Bunina bodies but not skein-like inclusions and Lewy body-like inclusions/round inclusions after uptake of transferrin from the exterior to the interior of neurons. There are several possibilities: (1) there are proteins that interact with transferrin in the components of Bunina bodies, (2) the coexistence is simply chance, (3) some relationship of transferrin to cystatin C is present. It is important to elucidate the origin of Bunina bodies or what organelle of the neurons is associated with Bunina bodies. Since Bunina bodies were also immunopositive for cystatin C [23], the third possibility is conceivable. The presence of both transferrin and cystatin C was demonstrated in the paper by Vannier-Santos and coworkers who concluded that the formation of inclusion vesicles displaying a large number of normal and polymorphic electron-dense acidocalcisome-like organelles surrounded by profiles of the endoplasmic reticulum in *Leishmania amazonensis* was induced by terbinafine, a sterol biosynthesis inhibitor [28]. In their report, acidocalcisome-like organelles showed acid phosphatase positive and the presence of endocytic tracers horseradish peroxidase (fluid-phase endocytosis), gold-labeled transferrin (receptor-mediated endocytosis), and gold-labeled cystatin C, suggesting the association of these organelles with the endosomal–lysosomal pathway. Furthermore, gold-labeled transferrin endocytosed is delivered to lysosomes. The components of Bunina bodies including both transferrin and cystatin C could be partially related to those of inclusion vesicles observed in *Leishmania*. Although the mechanism underlying these processes remains to be clarified, it is likely that Bunina bodies-specific factors, not affecting skein-like inclusions and Lewy-body-like inclusions/round inclusions, might be involved in the formation of Bunina bodies and accumulation of transferrin and cystatin C.

With respect to basophilic inclusions, three different types of transferrin immunoreactivities were observed. These results may be caused by the sequence of protein aggregation. At the beginning of the formation of basophilic inclusions, transferrin would not be present in the inclusions. Reversely, transferrin would be there only at the early stage of the formation. The findings concerning different proportions of p62 and ubiquitin were also reported in the dentate gyrus of the patients with ALS-D [20], neurofibrillary tangles in Alzheimer's disease [8], hyaline bodies in liver carcinoma [27], and anterior horn cells in ALS [13], which might be similar to a different proportion of transferrin in basophilic inclusions in this study.

In summary, we showed for the first time that transferrin localizes in Bunina bodies and some of the basophilic inclusions. The mechanism by which transferrin accumulates in these inclusions remains to be clarified. It is thought that systemic derangements of transferrin may play a causal role in the pathogenesis of neurodegenerative diseases like ALS. Detailed examination of how the inclusions are formed, how the neurons survive by forming inclusions, and what effects the inclusions have on the remaining neurons could contribute to clarifying the pathogenesis of ALS.

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In vitro Peptide Immunization of Target Tax Protein Human T-Cell Leukemia Virus Type 1–Specific CD4$^+$ Helper T Lymphocytes

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Abstract

Purpose: Adult T-cell leukemia/lymphoma induced by human T-cell leukemia virus type 1 (HTLV-1) is usually a fatal lymphoproliferative malignant disease. HTLV-1 Tax protein plays a critical role in HTLV-1-associated leukemogenesis and is an attractive target for vaccine development. Although HTLV-1 Tax is the most dominant antigen for HTLV-1-specific CD8$^+$ CTLs in HTLV-1-infected individuals, few epitopes recognized by CD4$^+$ helper T lymphocytes in HTLV-1 Tax protein have been described. The aim of the present study was to study T-helper-cell responses to HTLV-1 Tax and to identify naturally processed MHC class II–restricted epitopes that could be used for vaccine development.

Experimental Design: An MHC class II binding peptide algorithm was used to predict potential T-helper cell epitope peptides from HTLV-1 Tax. We assessed the ability of the corresponding peptides to elicit helper T-cell responses by *in vitro* vaccination of purified CD4$^+$ T lymphocytes.

Results: Peptides $Tax_{191-205}$ and $Tax_{305-319}$ were effective in inducing T-helper-cell responses. Although $Tax_{191-205}$ was restricted by the *HLA-DR1* and *DR9* alleles, responses to $Tax_{305-319}$ were restricted by either DR15 or DQ9. Both these epitopes were found to be naturally processed by HTLV-1$^+$ T-cell lymphoma cells and by autologous antigen-presenting cells that were pulsed with HTLV-1 Tax$^+$ tumor lysates. Notably, the two newly identified helper T-cell epitopes are found to lie proximal to known CTL epitopes, which will facilitate the development of prophylactic peptide–based vaccine capable of inducing simultaneous CTL and T-helper responses.

Conclusion: Our data suggest that HTLV-1 Tax protein could serve as tumor-associated antigen for CD4$^+$ helper T cells and that the present epitopes might be used for T-cell-based immunotherapy against tumors expressing HTLV-1.

Human T-cell leukemia virus type 1 (HTLV-1) is a member of the mammalian type C oncovirus family and is the only known infectious agent etiologically associated with adult T-cell leukemia/lymphoma (ATLL; refs. 1, 2). Infection with this virus can also lead to a slowly progressive neurologic disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP; ref. 3). It is known that the majority of seropositive individuals remain throughout their lives as asymptomatic carriers due to diverse factors, such as genetic predisposition, the route of infection, and the presence of cytotoxic and helper T-lymphocyte responses that could play a role in the control of disease progression (4–10). Although exposure to this virus usually leads to a persistent infection, once ATLL develops, it progresses rapidly and becomes resistant to conventional chemotherapy, causing a high mortality rate (11). Therefore, it is felt that immunologic approaches, such as T-cell-based vaccines to treat or prevent the HTLV-1-associated malignancy, could be of value.

HTLV-1 possesses four main genomic regions—Gag, Pol, Env, and pX (12). The *pX* gene encodes the Tax 40-kDa transcriptional regulatory protein, which is known to interact with various cellular transcription factors promoting genetic mutations that inhibit apoptosis of infected host cell and lead to drive host cell proliferation and transformation. It has been reported that Tax is a dominant target antigen recognized by HTLV-1-specific CTLs from asymptomatic carriers, which are capable of killing HTLV-1$^+$ leukemic cells (6, 13, 14). The low frequency of HTLV-1 Tax–specific CTLs observed in ATLL patients probably contributes to HTLV-1-induced leukemogenesis. On the other hand, host immune responses against HTLV-1 tend to be higher in HAM/TSP patients than in ATLL patients (6, 7, 15). Together, these observations suggest that the Tax is a promising tumor-associated antigen (TAA) for the development of prophylactic vaccines for HTLV-I and that augmentation of

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Tax-specific CTLs in pre-ATL patients could protect them from progressing into ATLL (16).

Both neutralizing antibody and CTL responses could be critical for viral clearance and in eliminating viral-infected and transformed cells (17–19). Because Tax is expressed early in the infection and is essential for the replication and persistence of the virus, vaccines to stimulate Tax-specific T-cell responses would be useful to inhibit both virus replication and viral-induced transformation. Consequently, a large number of MHC class I–restricted CTL epitopes derived from the Tax protein have been identified (6, 13, 14, 20). However, we believe that the $CD4^+$ helper T lymphocytes (HTL) also play an important role in HTLV-1 infection because HTLs are required for clonal expansion of antibody-secreting B cells and induction and maintenance of optimal CTL responses (21, 22). Moreover, in some instances, HTLs can exhibit an effector function by directly recognizing and killing MHC class II^+ virus-infected or tumor cells that present peptide epitopes on their surface (23–26). Thus, studies of HTL responses against HTLV-1 Tax could be of interest and elucidating the corresponding MHC class II–binding peptide epitopes will be necessary for designing more effective vaccines than those only inducing CTL responses.

In the present study, we describe two novel MHC class II–restricted epitopes, HTLV-1 $Tax_{191-205}$ and HTLV-1 $Tax_{305-319}$, both capable of stimulating *in vitro* $CD4^+$ HTL responses from HTLV-1-naïve individuals. More importantly, the peptide-reactive HTLs were effective in directly recognizing HTLV-1-infected, MHC class II^+ T-cell lymphoma cell lines. In addition, the Tax-specific HTL recognized naturally processed antigen in the form of cell lysates prepared from HTLV-1^+ T-cell lymphoma cell lines or from lymphocytes from HAM/TSP patients presented by autologous antigen presenting cells (APC). Interestingly, our described HTL epitopes, $Tax_{191-205}$ and $Tax_{305-319}$, are located in close proximity to previously described HLA-B14 and HLA-A24-restricted CTL epitopes, respectively (15, 27, 28), which could facilitate the development of peptide vaccines capable of stimulating both CTLs and HTLs for the treatment/prevention of HTLV-1-associated ATLL.

Materials and Methods

Cell lines. EBV-transformed lymphoblastoid cells (EBV-LCL) were produced from peripheral blood mononuclear cells (PBMC) of HLA-typed volunteers using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection, Manassas, VA). Mouse fibroblast cell lines (L-cells) transfected and expressing individual human MHC class II molecules were kindly provided by Dr. Robert W. Karr (Pfizer Global R&D, New London, CT) and by Dr. Takehiko Sasazuki (Tokyo, Japan). The HTLV-I-infected T-cell lymphoma cell lines TL-Su, TCL-Kan, and HUT102 and T-cell leukemia cell line TL-Hir (HTLV-1 Tax negative) were supplied by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). The HTLV-1-infected T-cell lymphoma cell line OKM-2T was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). The Jurkat T-cell lymphoma cell line (HTLV-I negative) was purchased from American Type Culture Collection. MT2 is an HTLV-I-transformed T cell line that was kindly provided by Dr. Y. Hinuma (Institute of Virus Research, Kyoto University, Kyoto, Japan; ref. 29).

Synthetic peptides. Potential HLA-DR-restricted $CD4^+$ T-cell epitopes were selected from the amino acid sequence of the HTLV-I-Tax using algorithm tables for three HLA-DR alleles, *DRB1*0101*, *DRB1*0401*, and *DRB1*0701* (30). The predicted peptide epitopes were synthesized by solid phase organic chemistry and purified by high-performance liquid chromatography. The purity (>80%) and identity of peptides were assessed by high-performance liquid chromatography and mass spectrometry, respectively.

In vitro *induction of antigen-specific HTL lines with synthetic peptides.* The procedure selected for the generation of HTLV-I-Tax-reactive HTL lines using peptide-stimulated PBMCs from five healthy donors whose MHC class II alleles were HLA-DR1/15 DQ5/6, HLA-DR4/15 DQ1/4, HLA-DR4/9 DQ7/8, HLA-DR9/14 DQ5/9, and HLA-DR9/14 DQ7/9. This procedure has been previously described in

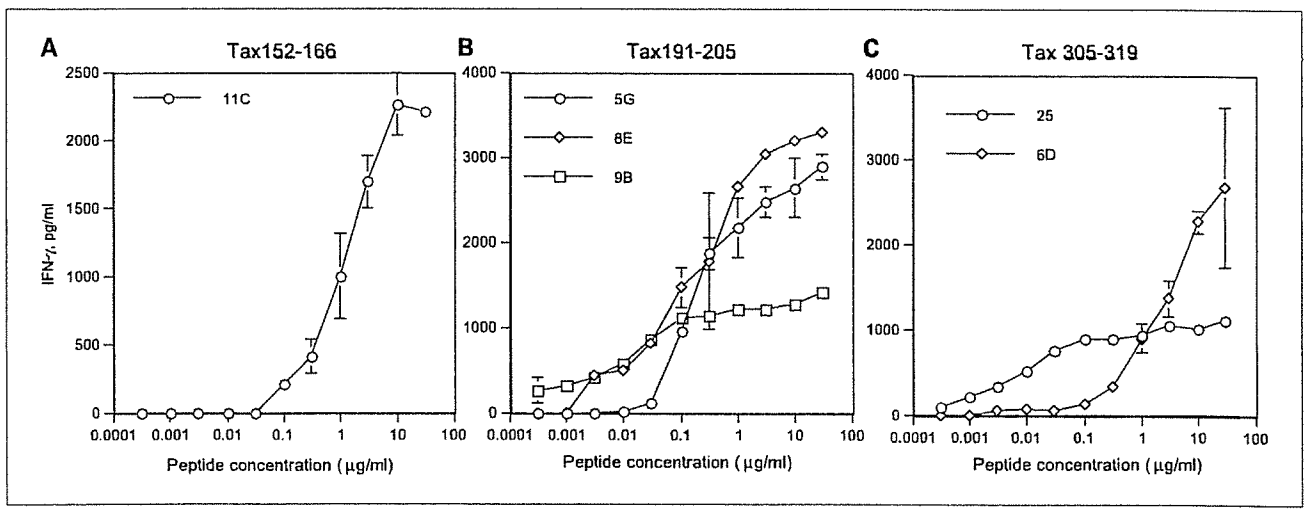

Fig. 1. Induction of HTL responses using predicted peptide epitopes derived from HTLV-1 Tax. HTL clones induced with peptide $Tax_{152-166}$ (*A*; clone 11C from DR4/9, DQ7/8 donor), peptide $Tax_{191-205}$ (*B*; clone 5G from DR1/15, DQ5/6 donor; clone 8E from DR4/9, DQ7/8 donor; and clone 9B from DR9/14, DQ7/9 donor), or peptide $Tax_{305-319}$ (*C*; clone 25 from DR4/15, DQ1/4 donor and clone 6D from DR9/14, DQ5/9 donor) were tested for their capacity to recognize autologous PBMC as APCs in the presence of various concentrations of peptide. Points, mean of triplicate determinations; bars, SD. Points without bars had SD <10% the value of the mean. Results are representative of at least two experiments that were done with the same samples.

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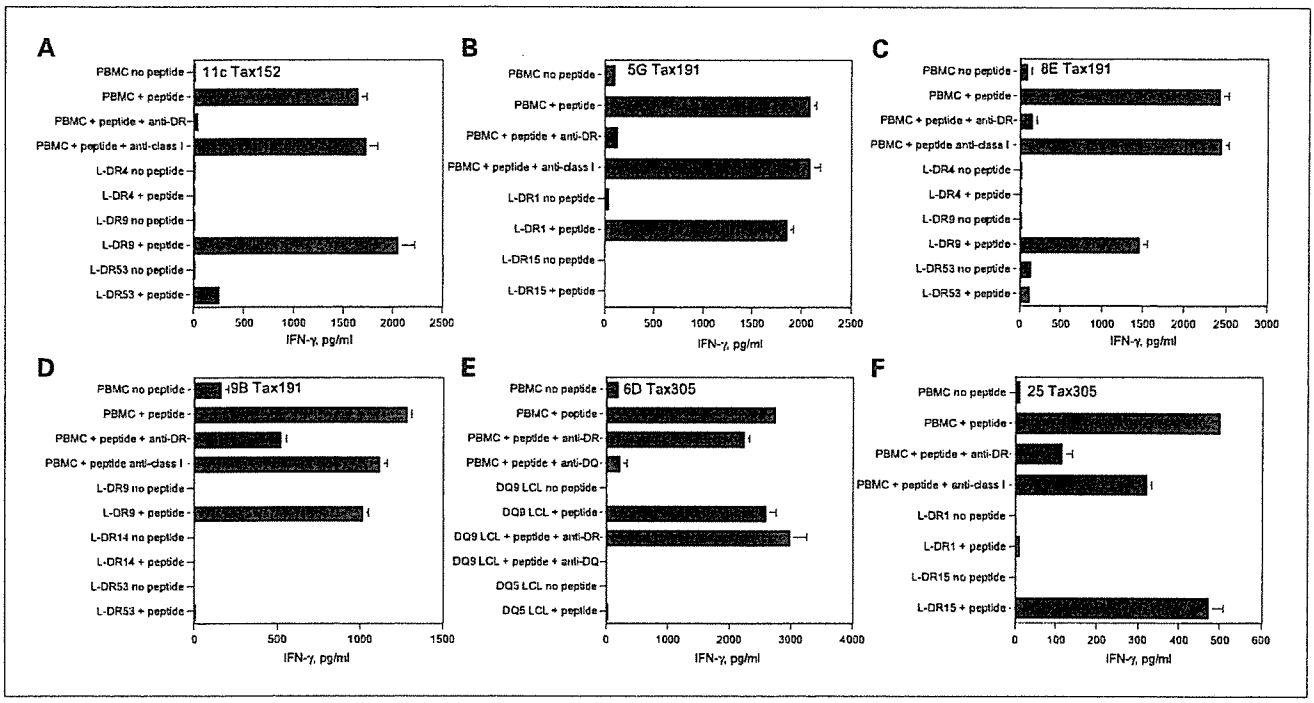

Fig. 2. MHC restriction analysis of peptide-reactive $Tax_{152-166}$ HTL (*A*), peptide-reactive $Tax_{191-205}$ HTL (*B-D*), and peptide-reactive $Tax_{305-319}$ HTL (*E* and *F*). MHC class II restriction molecules were identified using antibody blocking: anti-DR L243, anti-DQ SPV-L3, or anti-class I W6/32 (negative control) all used at 10 μg/mL. Experiments were done using irradiated peptide-pulsed autologous PBMCs as APC. In addition, HTL responses were also evaluated using various MHC-typed APCs (L cells transfected with individual HLA-DR genes or allogeneic EBV-LCLs homozygous for DQ molecules) to define the restricting MHC class II alleles. *A*, peptide $Tax_{152-166}$-reactive HTL clone 11C recognized antigen in the context of HLA-DR9. *B*, peptide $Tax_{191-205}$-reactive HTL clone 5G recognized antigen in the context of HLA-DR1. *C* and *D*, peptide $Tax_{191-205}$-reactive HTL clone 8E and HTL clone 9B recognized antigen in the context of HLA-DR9. *E*, peptide $Tax_{305-319}$-reactive HTL clone 6D recognized antigen in the context of HLA-DQ9. *F*, peptide $Tax_{305-319}$-reactive HTL clone 25 recognized antigen in the context of HLA-DR15. Columns, mean of triplicate determinations; bars, SD. Columns without bars had SD <10% the value of the mean. Results are representative of two experiments that were done with the same samples.

detail (31). Briefly, dendritic cells were produced in tissue culture from purified $CD14^+$ monocytes (using antibody-coated magnetic microbeads from Miltenyi Biotech, Auburn CA) that were cultured for 7 days at 37°C in a humidified CO_2 (5%) incubator in the presence of 50 ng/mL granulocyte macrophage colony-stimulating factor and 1,000 IU/mL interleukin-4. Peptide-pulsed dendritic cells (3 μg/mL for 2 hours at room temperature) were irradiated (4,200 rads) and cocultured with autologous purified $CD4^+$ T cells (Miltenyi Biotech) in 96 round-bottomed-well culture plates. One week later, the $CD4^+$ T cells were restimulated with peptide-pulsed irradiated autologous PBMCs, and 2 days later human recombinant interleukin-2 was added at a final concentration of 10 IU/mL. One week later, the T cells were tested for antigen reactivity using a cytokine release assay as described below. Those cultures exhibiting a significant response of cytokine release to peptide (at least 2.5-fold over background) were expanded in 24- or 48-well plates by weekly restimulation with peptides and irradiated autologous PBMC. Complete culture medium for all procedures consisted of AIM-V medium supplemented with 3% human male AB serum. All blood samples were obtained after the appropriate informed consent.

Measurement of antigen-specific responses with HTL lines. $CD4^+$ T cells (3×10^4 per well) were mixed with irradiated APC in the presence of various concentrations of antigen (peptides, tumor lysates), in 96-well culture plates. APC consisted of either autologous PBMC (1×10^5 per well), HLA-DR-expressing L cells (3×10^4 per well), MHC-typed EBV-LCLs (3×10^4 per well), T-cell lymphoma cell lines (3×10^4 per well), or autologous dendritic cells (5×10^3 per well). The expression of HLA class II molecules on tumor cell lines was evaluated by flow cytometry using anti-HLA class II monoclonal antibody (mAb), TÜ32, conjugated with fluorescein isothiocynate (BD Biosciences, San Jose, CA). Tumor cell lysates were prepared by three freeze-thaw cycles of 1×10^8 tumor cells that were resuspended in 1 mL serum-free RPMI 1640. Lysates were used as a source of antigen at 5×10^5 cell equivalents/mL. Culture supernatants were collected after 48 hours for measuring antigen-induced lymphokine (IFN-γ) production by the HTL using commercially available ELISA kits (PharMingen, San Diego, CA). To show antigen specificity and MHC restriction, blocking of the antigen-induced proliferative response was assessed by adding anti-HLA-DR mAb L243 (IgG2a, prepared from supernatants of the hybridoma HB-55 obtained from the American Type Culture Collection), anti-HLA-DQ mAb SPV-L3 (IgG2a, Beckman Coulter, Inc., Fullerton, CA), or anti-HLA-A, B, C mAb W6/32(IgG2a, American Type Culture Collection). The effect of antigen-specific antibodies on the response of HTL to HTLV-1 Tax protein was investigated by adding anti-p40 HTLV-1 Tax mAb Lt-4 (IgG3; ref. 32). All antibodies were used at a final concentration of 10 μg/mL throughout the 48-hour incubation period. All assessments of ELISA were carried out at least in triplicate and results correspond to the mean values with SD.

Western blot analysis. One million tumor cells were washed in PBS and lysed in Laemmli buffer. The cell lysate was subjected to electrophoresis in a 4% to 12% NuPage bis-Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA) under reducing condition and then transferred to Immobilon-P (Millipore, Bedford, MA) membrane. The membrane was then blocked in PBS containing 0.01% Tween 20 and 5% nonfat dry milk for 1 hour at room temperature and incubated first with anti-p40 HTLV-1 Tax mAb Lt-4 at 1 μg/mL in blocker overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase–labeled goat anti-mouse IgG and subjected to the enhanced chemiluminescence assay using the ECL detection system (Amersham, Little Chalfont, Buckinghamshire, UK).

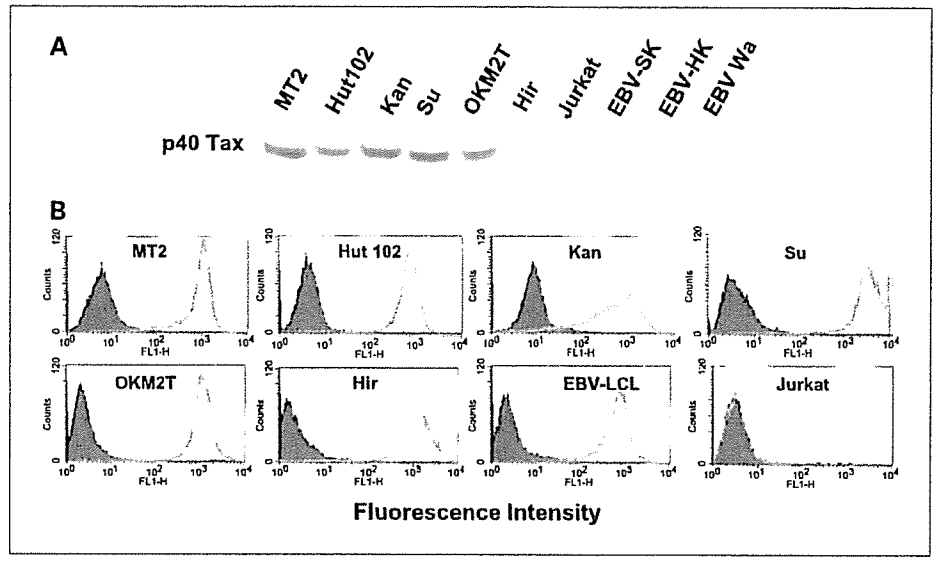

Fig. 3. Expression of HTLV-1 Tax protein and cell surface expression of MHC class II molecules in human tumor cell lines. *A*, Western blot analysis was done using a HTLV-1 Tax – specific mAb Lt-4 as described in Materials and Methods. HTLV-1 Tax protein was detected in the indicated HTLV-1-infected T-cell lymphoma cell lines but not in the T-cell leukemia cell lines Hir and Jurkat and in the EBV-LCLs. The HTLV-1 Tax protein has a mass of 40 kDa. *B*, the expression of HLA class II molecules on tumor cells was evaluated by flow cytometry using anti-HLA class II mAb Tu 39 conjugated with fluorescein isothiocynate (*thick-line open histograms*). Staining with the isotype-negative control (*filled histograms*). All tumor cell lines, except Jurkat T-cell lymphoma, expressed high levels of surface MHC class II molecules.

Results

Prediction and selection of potential HTL epitopes for HTLV-1 Tax protein. First, we used the MHC class II peptide binding algorithm developed by Southwood et al. (30) to select potential peptides from the Tax protein that would bind to HLA-DR1, HLA-DR4, and HLA-DR7. With this algorithm, we succeeded in defining the HTL epitopes from multiple TAAs such as proteins that are overexpressed by epithelial tumors, melanomas, and oncogenic viruses (23–25, 31, 33–37). In addition, the work by Southwood et al. (30) reported that some peptides that score high for the DR1, DR4, and DR7 algorithms also have the capacity to bind to additional MHC class II alleles such as DR9, DR13, DR15, and DR52, indicating that this algorithm is effective for identifying highly promiscuous MHC class II binders. In support of this, we reported that HTL responses induced by peptides predicted by this algorithm to be promiscuous MHC class II binders were restricted by DR9, DR15, DR16, DR52, DR53, DQ2, and DQ6. In this study, this prediction system could select eight peptide sequences from HTLV-1 Tax protein as potentially promiscuous MHC class II–restricted T-cell epitopes (data not shown). When examining the position that these peptides occupy within the Tax protein sequences, some peptide sequences were located near previously described CTL epitopes. Specifically, the predicted peptide Tax_{86-100} was found proximal to a previously described HLA-A2-restricted CTL epitope (Tax_{80-95}), peptide $Tax_{152-166}$ was found to overlap with another HLA-A2-restricted CTL epitope ($Tax_{151-165}$), peptide $Tax_{191-205}$ was found to lie proximal to an HLA-B44-restricted CTL epitope ($Tax_{181-195}$), and peptide $Tax_{305-319}$ was found to overlap with an HLA-A24-restricted CTL epitope ($Tax_{301-315}$; refs. 27, 28, 38). Because it would be advantageous for a single peptide vaccine to simultaneously elicit effective CTL and HTL responses, we decided to focus our efforts in studying those potential HTL epitopes that contained proximal CTL epitopes.

Isolation of HTLV-1 Tax peptide–reactive T-helper cells from healthy individuals. Peptides Tax_{86-100}, $Tax_{152-166}$, $Tax_{191-205}$, and $Tax_{305-319}$ were synthesized and evaluated for their ability to elicit T-helper responses by *in vitro* vaccination of PBMC from five healthy volunteers. Purified $CD4^+$ T cells were stimulated *in vitro* with individual each peptide pulsed autologous dendritic cells. One week later, after restimulating with γ-irradiated autologous PBMCs and peptides, microcultures were tested for their ability to produce IFN-γ upon stimulation with peptide-loaded autologous PBMCs. Positive microcultures that exhibited at least a 2.5-fold increase in IFN-γ production to peptide compared with in the absence of peptide were expanded in 48- or 24-well plate for further analysis, and in some cases T-cell clones were also isolated by limiting dilution. As shown in Fig. 1, three of the four peptides ($Tax_{152-166}$, $Tax_{191-205}$, and $Tax_{305-319}$) were able to elicit peptide-specific HTLs that responded to their corresponding peptide in a dose-dependent manner as presented by autologous APCs.

HLA restriction analysis of HTLV-1 Tax peptide–reactive HTLs. Peptide-reactive HTL clones were isolated by limiting dilution and were analyzed for their MHC class II restriction pattern. Peptide-induced lymphokine production was evaluated using a panel of HLA-DR-transfected mouse fibroblasts (L cells) or EBV-LCLs (homozygous for MHC class II), which were used as APCs. In addition, anti-HLA-DR (L243), anti-HLA-DQ (SPV-L3), or anti-HLA class I mAbs (W6/32) were used to inhibit the response of these HTL to antigen. The results presented in Fig. 2 show that peptide $Tax_{152-166}$ could be presented to the T cells in the context of HLA-DR9 (HTL clone 11C), and the recognition of peptide $Tax_{191-205}$ by the HTL clones was restricted by HLA-DR1 (for clone 5G) and HLA-DR9 (for clone 8E and 9B). HTL clones 6D and 25, which were elicited by peptide HTLV-1 $Tax_{305-319}$, responded to peptide presented by HLA-DQ9 and DR15, respectively. These results indicate that at least two of the three peptides ($Tax_{191-205}$ and $Tax_{305-319}$) behave as promiscuous HTL epitopes because more than one MHC class II allele can present them.

Recognition of HTLV-1-infected T-cell lymphoma cells by peptide-reactive HTLs. We proceeded to assess whether the peptide-reactive HTLs would be able to directly recognize intact HTLV-1-infected T-cell lymphoma cells that endogenously express the Tax gene product. This would signify that these peptide epitopes can be expressed on the MHC class II molecules of the tumor cells. Before performing these

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experiments, we first examined whether our HTLV-1-positive T-cell lymphoma cell lines expressed the Tax protein and cell surface MHC class II molecules. As shown in Fig. 3, all five HTLV-1-infected T-cell lymphoma cells, MT2 (DR4/15, DQ6/8), Hut102 (DR15, DQ6), Kan (DR4/9, DQ8/9), Su (DR9/15, DQ6/9), and OKM2T (DR1, DQ5) expressed the HTLV-1 Tax protein and surface HLA class II molecules. On the other hand, T-cell leukemia cell line Hir (DR4/9, DQ6/9) and the EBV-LCLs all expressed cell surface MHC class II molecules but did not express the Tax protein. The Jurkat T-cell lymphoma was negative for both HLA class II and Tax, allowing us to use some of these cell lines as negative control APCs. The data presented in Fig. 4 indicates that HTL clones reactive with peptides $Tax_{191-205}$ and $Tax_{305-319}$ were effective in directly recognizing the MHC class II matched, HTLV-1 Tax–expressing T-cell lymphoma cell lines. These HTL clones did not react with autologous EBV-LCLs, indicating that the response was antigen specific. Moreover, the recognition of HTLV-1$^+$ T-cell lymphoma cells by these HTLs was inhibited by the corresponding anti-HLA-DR mAb (for HTLs 5G, 8E, 9B, and 25) or anti-HLA-DQ mAb (for HTL 6D), confirming that antigen recognition is through the presentation of peptide by MHC class II molecules (Fig. 5). However, our results showed that $Tax_{152-166}$-reactive HTL clone 11C did not recognize HTLV-1-infected T-cell lymphoma cells, suggesting that this epitope is not processed and presented by intact HTLV-1$^+$ tumor cells (data not shown).

Indirect recognition of naturally processed viral antigen by autologous dendritic cells. We have observed that some peptide-elicited HTLs can only respond with peptide-pulsed APCs but not with APCs that are fed protein or tumor lysates that requires antigen processing via the MHC class II endocytic pathway. Thus, we proceeded to evaluate whether the HTLV-1 Tax peptide-reactive HTLs would be able to recognize the naturally processed viral antigen. These experiments were done using autologous dendritic cells as APCs that were fed with freeze/thaw lysates derived from HTLV-1 Tax$^+$ T-cell lymphomas. As shown in Fig. 6A and C, both the $Tax_{191-205}$-reactive HTL line 9B and $Tax_{305-319}$-reactive HTL clone 25 responded efficiently to autologous dendritic cells pulsed with lysates from HTLV-1$^+$ T-cell lymphoma cells (MT2, Hut102) but not with dendritic cells pulsed with lysates from Jurkat cells (HTLV-I negative). In addition, these responses were inhibited by anti-HLA-DR mAb treatment, indicating that both $Tax_{191-205}$ and

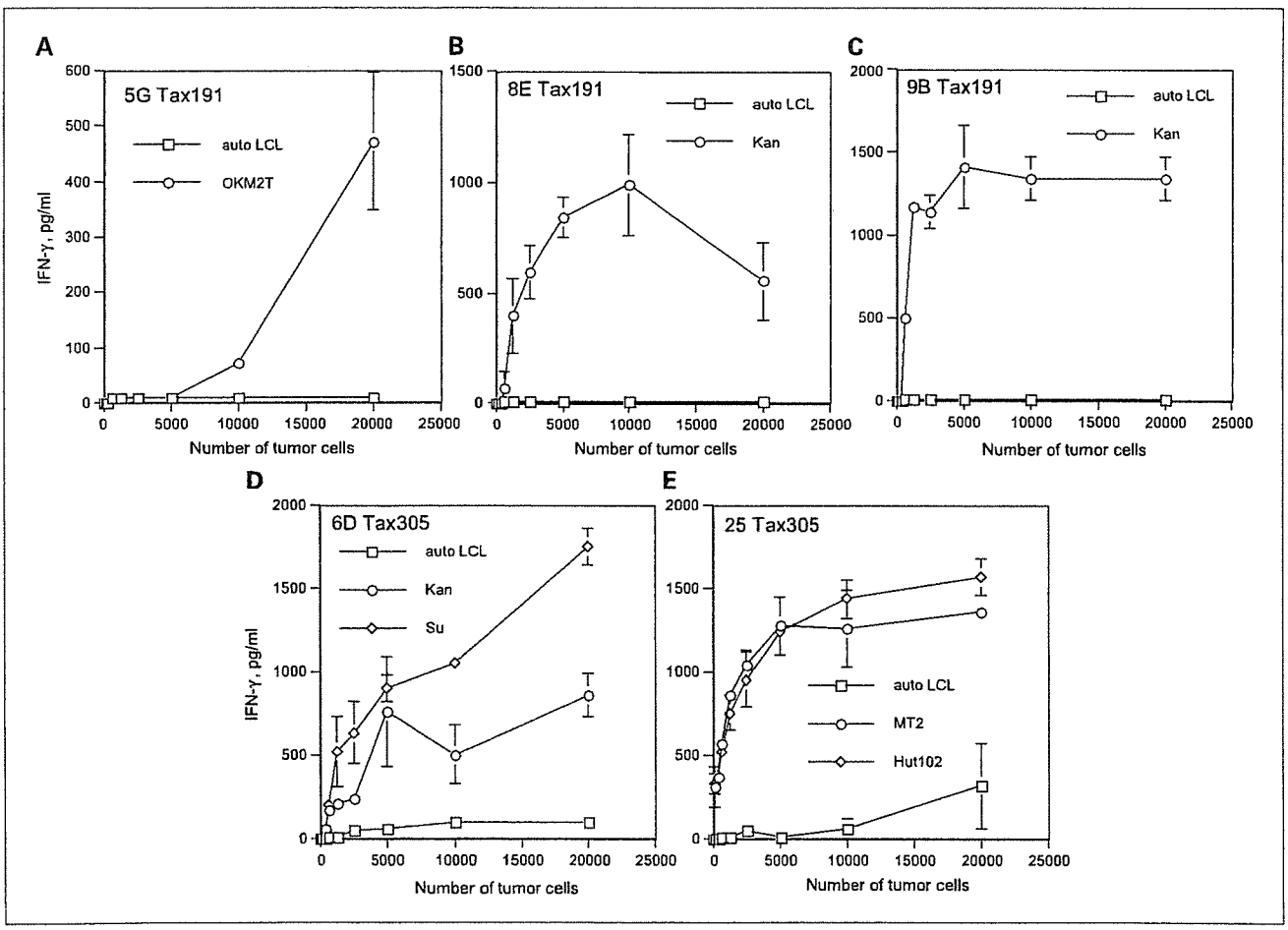

Fig. 4. Antitumor reactivity of the peptide $Tax_{191-205}$-specific HTLs 5G, 8E, and 9B (*A-C*) and peptide $Tax_{305-319}$-specific HTLs 6D and 25 (*D* and *E*). HTLs (3×10^4 per well) were tested at various APC numbers for their capacity to recognize the HTLV-1-positive T-cell lymphoma cells and autologous EBV-LCLs (HTLV-1 negative). The peptide-specific HTLs secreted IFN-γ as the result of recognizing antigen in the MHC class II – matched HTLV-1$^+$ T-cell lymphoma cell lines but not HTLV-1-negative autologous EBV-LCLs. Points, mean of triplicate samples; bars, SD. Points without bars had SD <10% the value of the mean. Results are representative of two experiments that were done under the same conditions.

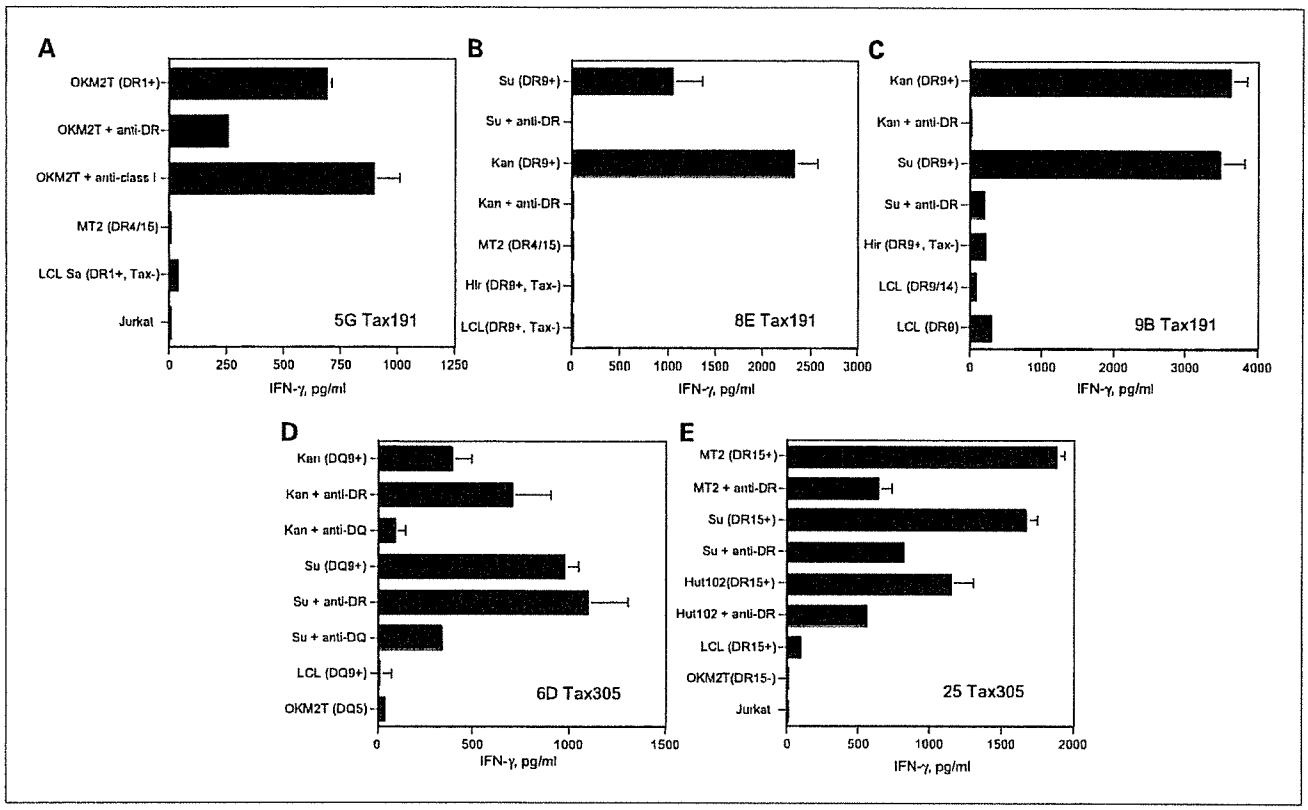

Fig. 5. Antigen specificity of direct tumor recognition by HTLV-1 Tax – reactive HTLs. The peptide $Tax_{191-205}$-reactive HTLs 5G, 8E, and 9B (*A-C*) and peptide $Tax_{305-319}$-reactive HTLs 6D and 25 (*D* and *E*) produced IFN-γ as the result of recognizing antigen on restricted HLA class II allele – positive HTLV-1-infected T-cell lymphoma cell line presenting the naturally processed epitope. Production of IFN-γ was inhibited by anti-HLA-DR mAb L243 (*A-D*) or anti-HLA-DQ mAb SPV-L3 (*E*) and could not be detected using HTLV-1-negative EBV-LCLs, HLA class II fully allogeneic HTLV-1$^+$ T-cell lymphoma cell lines, and HTLV-1-negative HLA class II Jurkat T cell lymphoma. Columns, means of triplicate determinations; bars, SD. Columns without bars had SD <10% the value of the mean. Representative of two experiments that were done under the same conditions.

$Tax_{305-319}$ were presented by MHC class II surface molecules. On the other hand, $Tax_{152-166}$-reactive HTL clone 11C, $Tax_{191-205}$-reactive HTL clones 5G and 8E, and $Tax_{305-319}$-reactive HTL clone 6D were not able to recognize the naturally processed antigen from tumor lysates presented by the autologous dendritic cells (data not shown). In these experiments, we observed that the T-cell responses to the tumor lysates could be substantially enhanced by the addition of anti-HTLV-1 Tax mAb, Lt-4 (Fig. 6B and D), presumably by increasing the delivery of antigen to the APCs via the Fc receptor – mediated endocytosis of immune complexes containing the relevant viral protein (39, 40). The enhancement by the anti-Tax mAbs was antigen specific because no effects were observed with lysates from Jurkat.

Recognition of naturally processed viral epitope from lysates prepared from HAM/TSP patient's PBMC by autologous dendritic cells. The results presented above, demonstrating indirect recognition of naturally processed antigens via autologous dendritic cells, were obtained using lysates derived from HTLV-1-infected cultured cell lines. We also examined whether T-cell epitopes $Tax_{191-205}$ and $Tax_{305-319}$ could be generated using lysates derived from "primary HTLV-1$^+$ PBMC" as source of antigen. It has been noted that expression of HTLV-1 viral antigens, including Tax in HTLV-1 infected cells, could increase by *in vitro* culture (10, 26, 41). Moreover, it is known that the expression of Tax mRNA in PBMCs isolated from HAM patients reaches a maximum at 24 hours after *in vitro* culture (41). Thus, we cultured PBMCs of patients with HAM for 24 hours before preparing the lysates. First, we confirmed by Western blot analysis that the lysates from PBMC derived from two HAM patients (HAM1, HAM2) expressed the HTLV-1 Tax protein (Fig. 7A). On the other hand, the Tax protein was not detected in lysates derived from PBMC of HTLV-1 naïve individuals. The HTLV-1$^+$ MT2 cells served as a positive control. Next, we assessed whether the $Tax_{191-205}$ and $Tax_{305-319}$-specific HTLs would be able to recognize the autologous dendritic cells pulsed with lysates prepared from HAM patient's PBMCs. Figure 7B and C shows that the autologous dendritic cells that were fed with PBMC lysates from both HAM1 and HAM2 patients were effective in stimulating the responses of the HTLs in an antigen-specific manner.

Discussion

CTLs have been proposed to be main effector cells against many pathogenic viruses, including HTLV-1. Thus, a large number of CTL epitopes derived from components of HTLV-1, such as the Tax, Env, Gag, and Pol proteins, have been identified and some of these are being considered as potential subunit peptide-based vaccine candidates (6, 13, 14, 20, 42).

However, recent findings indicate that the presence of antigen-specific $CD4^+$ helper T lymphocytes is necessary for the optimal induction and maintenance of antigen-specific CTL and for the development of effective antibody responses. Using an experimental animal model for HTLV-1, rats that were inoculated with HTLV-1-infected T-cell lymphoma cells exhibited antiviral $CD4^+$ T helper responses, indicating that the HTLV-1-infected tumor cells were taken up by APCs and that the naturally processed epitopes were effectively processed and presented via the MHC class II pathway (21). Moreover, it has been reported that HTLV-1-reactive $CD4^+$ T cells are present in HAM/TSP patients and the T-helper cells produce Th1 cytokines (interleukin-2, IFN-γ) in responses to antigen stimulation (10, 26). In view of the above, it is clear that the identification of MHC class II–restricted tumor and viral HTL epitopes in addition to CTL epitopes will be a critical step in the development of effective vaccines for this virus. As a key to the above problem, we previously showed that three HTLV-1 Env peptides—$Env_{196-210}$, $Env_{317-331}$, and $Env_{384-398}$—induced HTLs that recognized intact HTLV-1^+ T-cell lymphoma cells *in vitro* and that some HTLs were able to directly recognize and lyse HTLV-1^+ T-cell lymphoma cells (35). Interestingly, HTL epitopes $Env_{196-210}$ and $Env_{384-398}$ are located closely to previously described antibody epitopes (17, 18, 43).

In the present study, we identified the two naturally processed MHC class II–restricted HTL epitopes from the HTLV-1 Tax protein ($Tax_{191-205}$ and $Tax_{305-319}$). A number of groups have presented evidence that HTLV-1 Tax is a major target for HTLV-1-specific CTLs (6, 13). Yamano et al. (44) indicated that CTL activity against Tax is predominantly detected in HAM/TSP patients and in some patients, the frequency of Tax-specific CTLs can be as high as 30% of all $CD8^+$ T cells in peripheral blood. Moreover, recent work by Harashima et al. (28) reported that after successful treatment by hematopoietic stem cell transplantation of an ATLL patient, most of CTL activity (>60%) was toward Tax. In general, although HAM/TSP patients suffer from chronic neurologic disorders, those patients exhibiting high CTL responses against HTLV-1 Tax rarely progress to ATLL. On the other hand, the low levels of Tax-specific CTLs observed in ATLL patients and the observation that these CTL are capable of killing leukemic cells *in vitro* led us to hypothesize that the absence of Tax-reactive CTLs in some ATLL patients may be associated with leukemogenesis (5–7). Furthermore, a recent report showed that when HTLV-1-specific CTLs were induced in ATLL patients, it was difficult to expand them *in vitro*, suggesting the presence of an immunosuppression or tolerance effect. Alternatively, the cause could be a lack of $CD4^+$ T helper cells, which are required for effective CTL expansion (45). It should be mentioned that although $CD4^+$ T cells are main viral reservoir of HTLV-1, some reports indicate that HTLV-1-specific $CD4^+$ T cells in HAM/TSP patients are not themselves infected with transcriptionally active HTLV-1 virus, implying that they would not be targeted by HTLV-1-specific CTL (26).

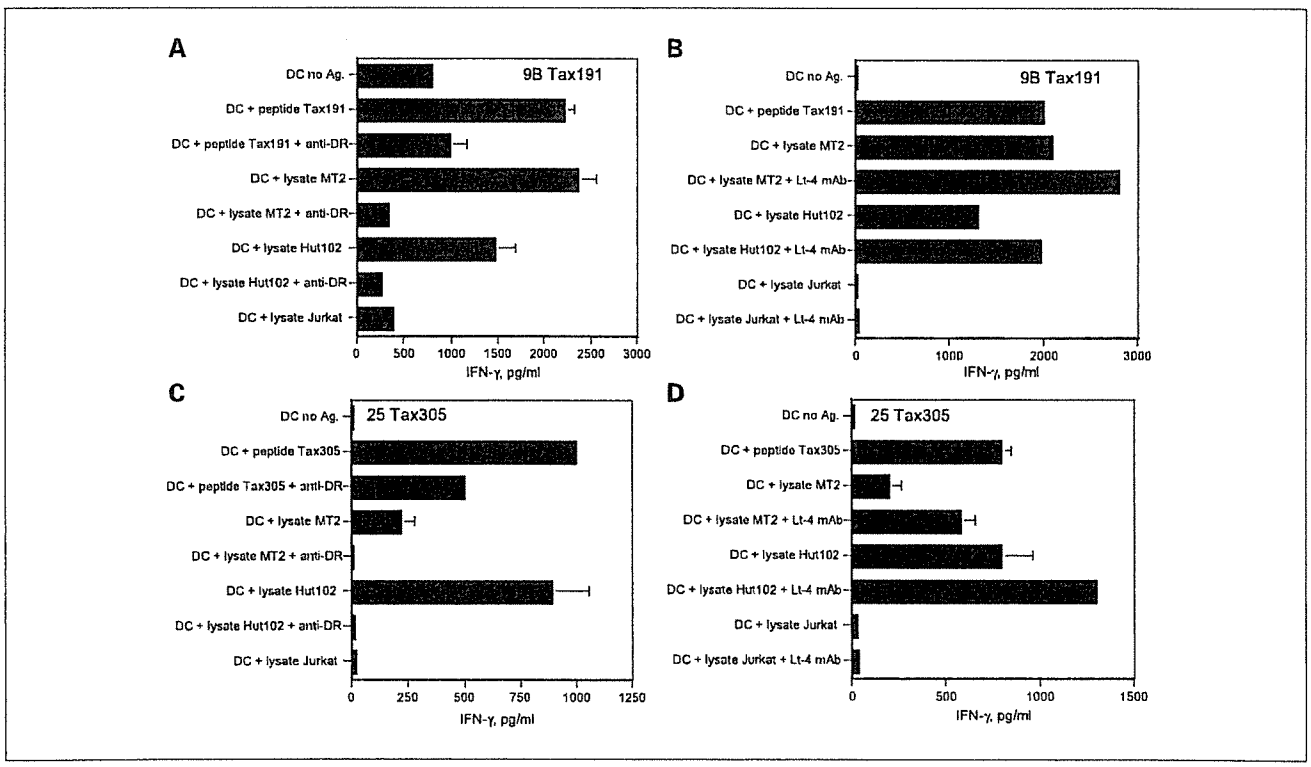

Fig. 6. HTLV-1 Tax–reactive HTL recognize naturally processed exogenous antigen presented by autologous dendritic cells. The peptide $Tax_{191-205}$-reactive HTL line 9B (*A*) and peptide $Tax_{305-319}$-reactive HTL clone 25 (*C*) were able to recognize the HTLV-1-positive T-cell lymphoma cell lysates (MT2, Hut102) but not HTLV-1-negative Jurkat T-cell lymphoma cell lysate presented by autologous dendritic cells. In addition, antigen recognition in all cases was inhibited by anti-DR mAb L243 at 10 μg/mL. The responses of these HTLs to the naturally processed antigen derived from tumor lysate were enhanced by adding HTLV-1 Tax–specific mAb Lt-4 at 10 μg/mL using tumor lysates from HTLV-1^+ MT2 or Hut102 but not HTLV-1-negative Jurkat lysate (*B* and *D*). Columns, mean of triplicate determinations; bars, SD. Columns without bars had SD <10% the value of the mean. Representative of two experiments that were done under the same conditions and with the same samples.

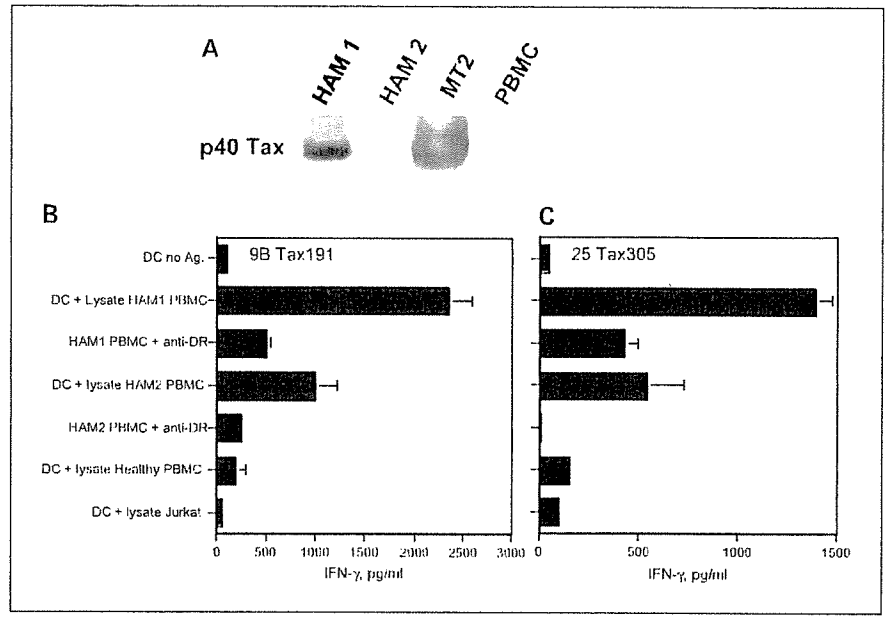

Fig. 7. Recognition of naturally processed HTLV-1 Tax protein derived from HAM patient's PBMC. *A*, HTLV-1 Tax protein content was evaluated by Western blot analysis using antibody Lt-4 specific for HTLV-1 Tax to determine whether the HTLV-1 Tax protein was present in PBMCs from two HAM patients (HAM1 and HAM2). The HTLV-1 Tax protein was detected in cell lysates prepared from both HAM patient's PBMC and MT2 cells (positive control HTLV-1$^+$ lymphoma) but not in PBMC from a healthy individual. *B* and *C*, the peptide $Tax_{191-205}$-specific HTL line 9B and peptide $Tax_{305-319}$-reactive HTL clone 25 recognized the lysates prepared from HAM patient's PBMC and the response was inhibited by anti-DR mAb L243 at 10 μg/mL. Results correspond to triplicate samples. Columns without bars had SD <10% the value of the mean. Representative of two experiments that were done with the same samples.

Recognition of naturally processed antigen has become the hallmark that a predicted T-cell epitope will be relevant for vaccine/immunotherapy development. Here, we have shown that T-cell responses induced with peptide $Tax_{191-205}$ by the DR1-restricted HTL 5G and the DR9-restricted HTL 8E were accompanied by effective direct recognition of HTLV-1-expressing T-cell lymphoma cell lines. However, both of these HTLs were not able to recognize autologous APCs that were fed with tumor cell lysates. In contrast, the DR9-restricted HTL 9B, also specific for peptide $Tax_{191-205}$, was able to respond with both HTLV-1-positive T-cell lymphoma cells and professional APCs that exogenously captured tumor lysate. Similarly, although the $Tax_{305-319}$-specific HTL 25 could recognize both HTLV-1 Tax–expressing T-cell lymphoma directly and antigen processed by autologous dendritic cells, the HTL 6D, which is specific for the same epitope, was able to react only with the HTLV-1 Tax–expressing tumor cell lines and not with the lysate-fed APCs. These apparently contradictory findings could be explained by differences in the affinity for antigen (Fig. 1B and C). Regardless, these results show that both $Tax_{191-205}$ and $Tax_{305-319}$ HTL epitopes can be processed endogenously by tumor cells and exogenously by professional APCs but that the recognition pattern may differ among various HTLs.

We showed that adding HTLV-1 Tax–specific mAb (Lt-4) with tumor lysates enhanced T-cell responses (Fig. 6). Dendritic cells express several receptors for the Fc portion of IgG (FcγR), which mediate internalization of antigen-IgG complexes and promote efficient MHC class II–restricted antigen presentation. In the past, we observed that HTL responses to hepatitis B virus antigen was enhanced by adding antibodies due to an enhancement of antigen delivery to APC by FcR-mediated endocytosis (39). It is known that antibodies to HTLV-1 Tax protein are detected in most HTLV-1-infected subjects (46). The production of specific antibodies could induce formation of immune complexes between antigens derived from virus-infected cells (or tumor cells), which could be efficiently taken up by FcR on APCs to more efficiently activate CD4$^+$ T cells *in vivo*.

Early studies by Ohashi et al. (47, 48) showed effective antitumor effects induced by HTLV-1 Tax–encoded DNA vaccination in a rat model system. More recently, the same group reported that immunization with oligopeptides from HTLV-1 Tax elicited CTL responses that could eradicate fatal HTLV-1-infected T-cell lymphomas (21). In these studies, it was shown that HTLV-1-specific CD4$^+$ helper T lymphocytes and CD8$^+$ CTLs collaborated to eradicate HTLV-1$^+$ T-cell lymphomas, indicating that CD4$^+$ helper T-cell responses are necessary for induction of effective CTL responses against HTLV-1-associated tumor (21). Because peptides are relatively easy to synthesize and safe to administer, a peptide-based T-cell vaccination strategy would be an attractive approach against HTLV-1-associated malignancies. One would expect that vaccines capable of activating both CTLs and HTLs should be more effective than vaccines that only target CTL responses. Because the two HTL epitopes described here, $Tax_{191-205}$ and $Tax_{305-319}$ lie proximal to previously described CTL epitopes (27, 28, 38), one could envision the possibility of using a single peptide of relatively small size (<25 to ~30 residues) to stimulate both CTL and HTL responses in individuals expressing the appropriate MHC class I and class II alleles.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Treatment of Japanese Restless Legs Syndrome Patients with Cabergoline: An Open Clinical Preliminary Trial

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Abstract

Objective To determine the effective dose of cabergoline in Japanese patients with restless legs syndrome (RLS).

Methods Six cases of idiopathic RLS and three of RLS with Parkinson disease (PD) participated in an open clinical preliminary trial. All cases were diagnosed based on the clinical criteria of the International RLS Study Group. Three RLS cases (1.3%) were detected out of 229 consecutive cases with PD. RLS severity was evaluated with International RLS Study Group (IRLSSG) Rating Scale Version 2.2 before and one year after the treatment with cabergoline.

Results For 6 idiopathic RLS patients, the IRLSSG questionnaire scores improved from 25.5±3.7 to 10.7±8.9 (p=0.028, Wilcoxon test) with 1 mg of daily cabergoline at the endpoint. For 3 RLS cases with PD, the score was 21.7±3.7 before the treatment, and RLS symptoms completely disappeared with 1 mg of cabergoline. One of RLS cases with PD required additional cabergoline later because of parkinsonism. No adverse event with cabergoline was reported in this study.

Conclusion One mg of daily cabergoline is effective in some Japanese patients of RLS.

Key words: restless legs syndrome, cabergoline, levodopa, dopamine agonist, Parkinson disease, clinical trial

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Introduction

Restless legs syndrome (RLS) is characterized by an urge to move the legs, usually accompanied or caused by an uncomfortable sensation in the legs. The urge to move or unpleasant sensations begins or worsens during periods of rest or inactivity, such as lying down or sitting. The urge to move or unpleasant sensations are relieved by movement, and are worse in the evening or at night than during the day or only occur in the evening or at night (1). The sensory and motor symptoms of RLS often result in nocturnal insomnia and chronic sleep deprivation. Treatment with levodopa usually alleviates symptoms, however, many patients with RLS develop rebound or augmentation (2). The direct-acting dopamine agonists, with a longer duration of action, provide an alternative to levodopa. Bromocriptine (3), pergolide (4-7), pramipexole (8-10), ropinirole (11-13) and cabergoline (14-16) have shown good efficacy in RLS and also have reduced the frequency of augmentation and rebound. All of those reports are from Western countries. RLS occurs in about 5-20% of the adult population in Western countries (1, 17, 18). Although RLS is a common disorder in Western countries, the prevalence of RLS has been reported to be quite low (0.1-0.6%) in an Asian population (19). At least one-third of cases have a positive family history in Western countries, suggesting an autosomal-dominant pattern of inheritance (20-22). However, no familial RLS has been reported to date in Asian countries. Taken together, there may be an ethnic susceptibility to RLS and/or potential genetic predisposition. To examine the effectiveness of dopamine agonists for Japanese patients with RLS, we administered cabergoline, a long-acting dopamine agonist, to RLS patients.

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Table 1. Patient Characteristics, Dosage and Treatment Response

Patient No	type of RLS	Age(years) / Gender	Duration of RLS (years)	Duration of treatment with cabergoline (years)	Cabergoline at endpoint / maximum dose during treatment	IRLSSG score (before)	IRLSSG score (one year after)
1	idiopathic	73/m	5	1	. mg/3 mg	24	14
2	idiopathic	76/f	22	3	1 mg/1 mg	26	0
3	idiopathic	89/f	12	1	1 mg/3 mg	29	20
4	idiopathic	71/f	3	2	1 mg/3 mg	37	20
5	idiopathic	63/m	3	2	1 mg/1 mg	22	0
6	idiopathic	79/f	3	1	1 mg/3 mg	15	9
7	RLS with PD(HY-I)	78/f	1	3	1 mg/1 mg	12	0
8	RLS with PD(HY-I)	62/f	2	3	1 mg/1 mg	38	0
9	RLS with PD(HY-III)	73/f	5	3	3 mg/3 mg	15	0

PD(HY-I) = Parkinson disease (Hohen-Yahr stage I), PD(HY-III) = Parkinson disease (Hohen-Yahr stage III)

Patients and Methods

Six idiopathic RLS patients and three RLS patients with Parkinson disease (PD) participated in this study. All patients gave informed consent and fulfilled the essential diagnostic criteria for RLS based on the International RLS Study Group (IRLSSG) (1).

Three RLS cases (1.3%) were detected out of 229 serial cases with PD. When two patients with PD (Nos. 7 and 8 in Table 1) first came to our hospital, they did not complain of PD symptoms (i.e., gait disturbance, bradykinesia, or tremor) but rather, RLS symptoms. On neurological examination, they had unilateral mild cogwheel rigidity of the wrist, mild bradykinesia and minimal hand tremor at rest. We diagnosed them with RLS with Parkinson disease (Hohen-Yahr stage I).

No patient had been treated with dopamine agonists, levodopa, benzodiazepines, opioids or other psychiatric drugs at least 4 weeks prior to this study. Because there has been no direct comparison among the agonists for the treatment of RLS, we selected cabergoline because it has the longest half-life among the available agonists. To prevent peripheral dopaminergic side-effects, cabergoline was started with a minimal dose (0.25 mg) in the evening. The dose was increased in steps of 0.25-1.0 mg up to 3 mg until RLS symptoms clearly improved.

We translated the questionnaire of the IRLSSG Rating Scale (IRLS) Version 2.2, which was developed by the IRLSSG to evaluate the effect of treatment (23), into Japanese. We used the abbreviated Japanese questionnaire for our clinical practice. RLS severity was evaluated with IRLS Version 2.2 before and one year after the treatment with cabergoline. Any adverse events, including augmentation, were monitored by phone or reported when the patients saw their doctor. Wilcoxon test was used for statistical analysis.

Results

The data for all patients is summarized in Table 1. No adverse events were reported with cabergoline, and no patient withdrew from this study. The age was 74±8.3 (mean±SD) years, and the duration of RLS symptoms was 7.3±6.4 (mean±SD) years.

For 6 idiopathic RLS patients, the IRLSSG questionnaire scores improved from 25.5±3.7 to 10.7±8.9 ($p=0.028$, Wilcoxon test). Two idiopathic RLS patients (Nos. 2 and 5 in Table 1) reported that RLS disappeared within a week with the minimum daily dose of cabergoline (0.25 mg). These 2 patients later required an increase of dosage up to 1.0 mg of cabergoline to control RLS symptoms. There was no difference in effectiveness on RLS symptoms in the range of 1.0-3.0 mg of cabergoline for the other 4 idiopathic RLS patients (Nos. 1, 3, 4 and 6 in Table 1), 1.0 mg of cabergoline was selected as the endpoint.

For 3 RLS cases with PD, the score was 21.7±3.7 before the treatment with cabergoline, and RLS symptoms completely disappeared at the endpoint. The effective daily dosage of cabergoline for RLS symptoms at the endpoint was 1.0 mg. One of the RLS cases with PD (No. 9) required 3.0 mg of cabergoline later because of PD symptoms.

Discussion

IRLSSG questionnaire scores improved significantly, indicating that cabergoline was effective for RLS symptoms in a patient population in Asia, where RLS is observed less frequently than in Western countries. Although there may be genetic/ethnic differences in RLS between Western countries and Asian countries, dopamine agonists would be one of the effective treatments for RLS patients across races. The use of standardized diagnostic criteria applied in this study will help to determine the effectiveness of treatments for RLS. Cabergoline has the longest mean elimination half-life (up to 65 hours) among the dopamine agonists (14, 16). Cabergoline needs to be given only once daily because of its long half-life. The long duration of action of cabergoline results in sustained stimulation of the dopamine receptors, mimicking normal physiological dopaminergic stimulation. Cabergoline has the beneficial effects of a sustained dopamine agonist on idiopathic and PD-related RLS, while levodopa-related augmentation is possibly due to the short half-life of levodopa (2).

The striatonigral dopaminergic system has been implicated in RLS, by neuropharmacological data including PET and SPECT studies. However, the pathogenesis remains unknown. Anticonvulsants including clonazepam, opioids and benzodiazepines are considered the drugs of choice as well

as dopaminergic drugs (24). When dopaminergic drugs do not show a sufficient effect on RLS symptoms, other drugs, such as anticonvulsants, benzodiazepines or opioids, should be considered.

The prevalence and severity of RLS increase with aging, so that RLS is of importance particularly in geriatric patients. RLS may be underdiagnosed, probably because the initial symptoms are mild, without objective abnormality on neurological examination and are commonly thought to be psychogenic (25). We should pay more attention to the characteristic RLS symptoms because RLS usually leads to sleep disturbance and a decreased quality of life, especially for geriatric patients (26).

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Edaravone Diminishes Free Radicals from Circulating Neutrophils in Patients with Ischemic Brain Attack

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Abstract

Objective Treatment with a free radical scavenger could be a new option for ischemic brain attack, however, little is known about the alteration of oxidative stress markers induced by edaravone, a novel free radical scavenger, in human ischemic brain attack.

Methods We investigated the effects of edaravone on the oxidative stress markers in patients with ischemic brain attack. Twenty-one patients with ischemic brain attack and 19 controls were enrolled in this study. Blood samples were obtained just before and soon after the first administration of edaravone (30 mg) or ozagrel (40 mg). Intracellular reactive oxygen species of neutrophils were measured using 6-carboxy-2', 7'-dichlorodihydrofluorescin diacetate and a fluorescence-activated cell sorter. Superoxide from neutrophils, induced by phorbol myristate acetate (PMA), was determined by luminol-amplified chemiluminescence assay.

Results Treatment with 30 mg of edaravone significantly decreased the intracellular reactive oxygen species (ROS) of neutrophils (Wilcoxon test, p=0.0001) and PMA-induced superoxide produced by neutrophils (Wilcoxon test, p=0.001). Ozagrel did not alter the intracellular ROS or superoxide production of neutrophils.

Conclusion Reduction of intracellular ROS and suppression of superoxide production in neutrophils provide a potential explanation for the clinical efficacy of edaravone in patients with ischemic brain attack.

Key words: edaravone, free radical scavenger, reactive oxygen species, neutrophil, ischemic brain attack

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Introduction

Accumulating evidence implicates free radicals in the damage of ischemic brain attack, and free radical scavengers have been proposed as a new clinical strategy. A novel free radical scavenger, edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one), has been shown to prevent vascular endothelial injury *in vitro* (1) and cerebral damage following middle cerebral artery occlusion (2), delayed neuronal death in transient cerebral ischemia (3) and ischemic brain edema in rats (4, 5). Edaravone has been found to inhibit activation of the lipoxygenase pathway in the arachidonic acid cascade (6) and peroxidation of the phosphatidylcholine liposomal membrane (7), as well as to scavenge hydroxyradicals.

The clinical efficacy of edaravone on ischemic brain attack has been demonstrated by significant improvement in the functional outcome in a randomized, placebo-controlled, double-blind study (8). However, to date no data is available on the alteration of oxidative stress markers due to edaravone in human ischemic brain attack. We investigated the effects of edaravone on oxidative stress markers of circulating neutrophils in patients with ischemic brain attack.

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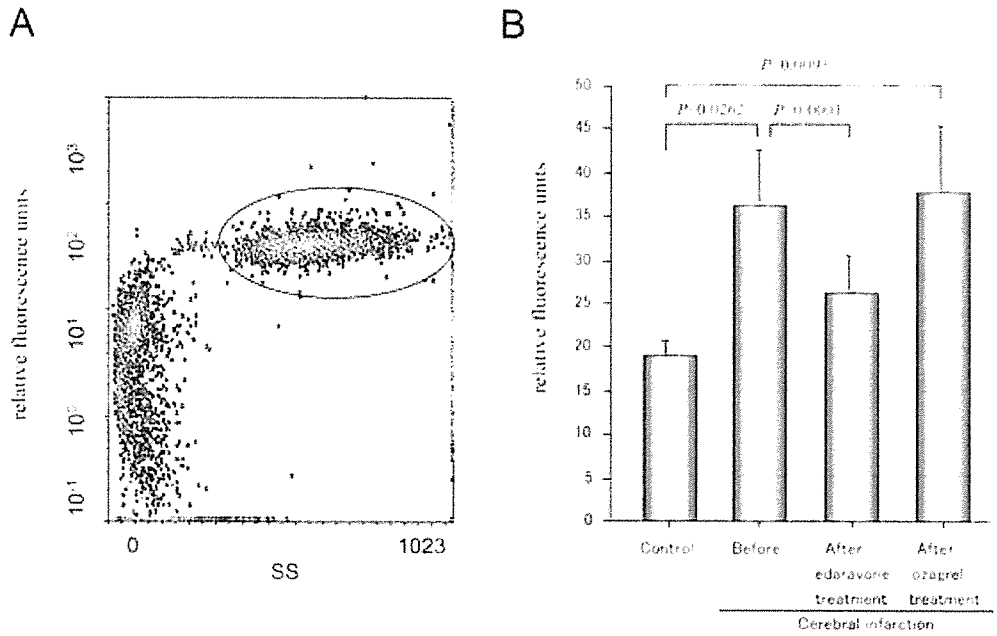

Figure 1. Intracellular reactive oxygen species of circulating neutrophils in patients with ischemic brain attack and controls. A: Typical flow cytogram of neutrophils (surrounded by an oval). Intracellular reactive oxygen species are presented as relative fluorescence units, i.e., the median channel value of the fluorescing cell population. SS = side scatter dot plot. B: Relative fluorescence units of neutrophils before and after treatment with 30mg of edaravone or 40 mg of ozagrel.

Patients and Methods

Twenty-one patients (14 males and 7 females, mean±SD; 71±2 years old, range 52-88) with ischemic brain attack and 19 untreated healthy controls (11 males and 7 females, mean ±SD; 51±4 years old, range 23-79) were enrolled in this study under informed consent. Edaravone treatment was started for the patients within 24 hours after the onset of ischemic brain attack. Patients were administered 30 mg of edaravone i.v. over 1 hour twice a day for 3-10 days. Ten patients were treated with 40 mg of ozagrel, a thromboxane A2 synthase inhibitor, over 1 hour before the treatment with edaravone. Blood samples were obtained just before and soon after the first administration of edaravone or ozagrel. Glycerol was used for the patients after the first treatment with edaravone.

To measure intracellular reactive oxygen species of neutrophils, venous peripheral blood was incubated in NH_4Cl (8.26 g/L) $KHCO_3$ (1.0 g/L) and tetrasodium ethylenediaminetetraacetic acid (0.037 g/L) in Chelex 100-treated water (pH 7.3) for 10 min to lyse erythrocytes, washed with ice-cold 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer and then centrifuged at 1,000 g at 4℃ for 5 min. The supernatant was discarded. Neutrophils were resuspended in 1 ml HEPES buffer, and 6-carboxy-2', 7'-dichlorodihydrofluorescin diacetate (CDCFH-DA;Molecular Probes, Netherlands) (10 μM final concentration) was added to each sample tube; and the tubes were incubated at 37℃ for 30 min before data acquisition by flow cytometry. A fluorescence-activated cell sorter (EPICS XL/XL-MCL System II v3.0, Becton Dickinson) and WinMIDI 28 software were used for acquisition and analysis of data. The stability of instrumental settings including spectral compensations was checked regularly with fluorescent beads (Calibrite, Becton Dickinson). For each 1 ml aliquot of cell suspension, an appropriate electronic gate was generated, and 5,000 events were collected in live mode. Neutrophils were delineated in a forward/side scatter dot plot, and appropriate fluorescence histograms were developed. Fluorescence intensities are reported in relative fluorescence units, i.e., the median channel value of the fluorescing cell population (Fig. 1A).

To measure superoxide produced by neutrophils, neutrophils were resuspended in 1 ml of HEPES buffer by the above method and adjusted to the density of 2×10^5 /ml. Superoxide production by phorbol myristate acetate (PMA) was determined by luminol-amplified chemiluminescence assay using a luminometer (BLR-301, ALOKA, Japan). Neutrophils were stimulated with PMA (0.1 nmol/L), and the resulting light output was continuously recorded on a chart recorder. All results are expressed as kilo-counts per minute (kcpm) using the chemiluminescence emission peak (Fig. 2A).

Results

The controls showed no significant relationship between

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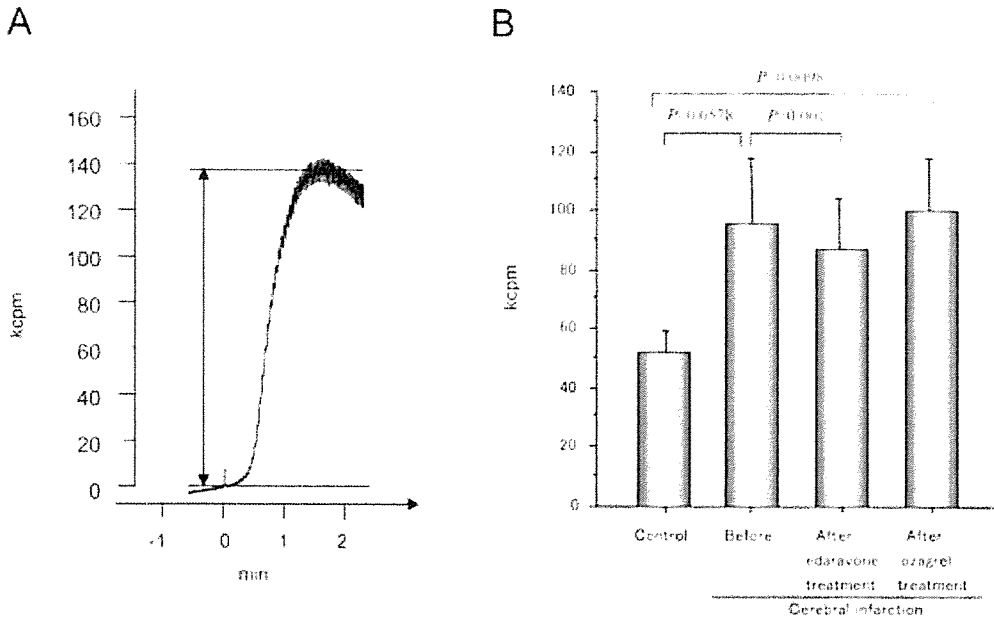

Figure 2. Superoxide production of circulating neutrophils in patients with ischemic brain attack and controls. A: Typical light output continuously recorded on a chart recorder, reflecting amount of superoxide produced by neutrophils stimulated by phorbol myristate acetate (PMA). B: Amount of superoxide produced by neutrophils stimulated by PMA before and after treatment with edaravone or ozagrel. All results are expressed as kilo-counts per minutes (kcpm) using the chemiluminescence emission peak.

age and relative fluorescence units of circulating neutrophils treated with CDCFH (simple regression analysis, p=0.1832) or between age and superoxide production by neutrophils (simple regression analysis, p=0.2406). The relative fluorescence units of circulating neutrophils in CDCFH in patients with ischemic brain attack before treatment with edaravone or ozagrel (36.8±5.9, mean±SE) was higher than that of controls (19.1±1.8) (Mann-Whitney U test, p=0.0262). The relative fluorescence units in CDCFH decreased after treatment with edaravone (26.8±3.8) (Wilcoxon test, p=0.0001), indicating that edaravone lowered the intracellular ROS of circulating neutrophils (Fig. 1B). In contrast, the relative fluorescence units in CDCFH showed no significant alteration after the treatment with ozagrel (38.3±7.2).

Amount of superoxide produced by neutrophils in patients with ischemic brain attack tended to be higher than those of controls (Mann-Whitney U test, p=0.0578). The superoxide production by neutrophils decreased after treatment with edaravone in patients with ischemic brain attack (Wilcoxon test, p=0.001; Fig. 2B). The amount of superoxide from neutrophils showed no significant alteration after the treatment of ozagrel. In one patient, we happened to obtain a blood sample one day before ischemic brain attack. The preliminary data showed that the superoxide produced by neutrophils after ischemic brain attack was increased to 135% of that before ischemic brain attack, indicating an increase in inducible superoxide from neutrophils after ischemic brain attack. No adverse events were observed in patients treated with 30 mg of edaravone.

Discussion

Brain damage in ischemic brain attack is closely related to inflammatory responses, especially infiltration of circulating neutrophils into ischemic tissue (9). Many studies show that inhibition of neutrophil accumulation greatly reduces infarct size (10, 11). Neutrophil accumulation in vessels requires interaction between several adhesion molecules, such as intracellular adhesion molecule (ICAM)-1. Interleukin (IL)-1β and tumor necrosis factor-α which upregulate ICAM (12) are increased in ischemic brain tissue. Ischemia induces the formation of IL-1, the subsequent expression of ICAM-1, and increased adherence of neutrophils. In ischemic brain tissue, neutrophil, cytokines, and adhesion molecules are closely related to each other.

Neutrophils are a potential source of ROS when activated during inflammatory responses. Free radicals from the neutrophils are produced via activation of NADPH oxidase in the cytoplasmic membrane. An ischemia and reperfusion animal model demonstrated that the major site of superoxide production is the activated leukocytes in the circulating blood (13). Superoxide and H_2O_2 have since been shown to directly trigger leukocyte activation and adhesion to vascular endothelium. Neutrophils might induce damage by causing local vascular occlusion, or they might initiate toxic reactions, including free radical production by NADPH oxidase, production of hypochlorous acid, or protease activation (14). Transgenic mice lacking NADPH oxidase showed reduction

of infarct size by 50%, however, selective elimination of either leukocyte or parenchymal NADPH oxidase did not reduce damage, suggesting both NADPH oxidases need to be eliminated to protect the brain from ischemia (15). A large part of free radical production is dependent on neutrophils, but it could be initiated by NADPH oxidation in parenchymal tissue or in the neutrophils (14). These findings suggest that inhibition of circulating neutrophils in ischemic brain attack could be effective to prevent ischemic brain injury.

We demonstrated here that edaravone diminished free radicals in circulating neutrophils in patients with ischemic brain attack, which could at least partially explain the clinical efficacy of edaravone on ischemic brain attack (8). We speculate that edaravone eventually protects the ischemic brain by scavenging free radicals from circulating neutrophils as well as from brain tissue.

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Immunoreactivities of p62, an ubiqutin-binding protein, in the spinal anterior horn cells of patients with amyotrophic lateral sclerosis

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Abstract

An ubiquitin-binding protein, p62, is one of the components of the ubiquitin-containing inclusions in several human neurodegenerative diseases. Amyotrophic lateral sclerosis (ALS) is characterized by the presence of skein-like inclusions, Lewy body-like inclusions, and basophilic inclusions in the remaining anterior horn cells, in which these inclusions contain ubiquitin, while the other characteristic inclusions of Bunina type are ubiquitin-negative. We examined the spinal cord from 28 ALS cases including two ALS with dementia and two ALS with basophilic inclusions, using antibody to p62. The results demonstrated that p62 localized in skein-like inclusions, Lewy body-like inclusions and basophilic inclusions. The number of p62-positive inclusions observed in the remaining anterior horn cells of each section was variable among the ALS cases. In contrast, Bunina bodies, that do not contain ubiquitin, were negative for p62. As far as we examined, the 11 non-ALS cases did not show any p62 immunoreactivities in the anterior horn cells. Our results suggested that p62 plays important roles in forming the inclusions and may be associated with the protection of the neurons from degenerative processes involving ubiquitin.

Keywords: Amyotrophic lateral sclerosis; p62; Ubiquitin; Skein-like inclusions; Lewy body-like inclusions; Basophilic inclusions

1. Introduction

In general, cytoplasmic inclusions, abnormal protein aggregation, are often the hallmarks of certain human neurodegenerative diseases such as neurofibrillary tangles in neurons of patients with Alzheimer's disease and Lewy bodies in neurons of patients with Parkinson's disease. Amyotrophic lateral sclerosis (ALS) is neuropathologically characterized by loss of motor neurons and occurrence of Bunina bodies (BB) [1], skein-like inclusions (SLI) [2], and Lewy body-like inclusions (LBLI)/ round inclusions [3,4] in the remaining anterior horn cells of spinal cords. Moreover, ALS with onset before the age of 20 has been reported as a sporadic juvenile case, in which cytoplasmic basophilic inclusions (BI) [5] have been observed as one of the characteristic features. BB is immunohistochemically positive for cystatin C [1], while SLI and LBLI show ubiquitin-positive, tau-negative, cystatin C-negative, and α-synuclein-negative immunoreactivities. BI show a globular or fragmented appearance and are occasionally positive for ubiquitin with granular reaction. The mechanism of the formation of cytoplasmic inclusions remains unknown; therefore, the elucidation of the main constituent is very important to understanding the significance of these inclusions.

p62 was originally identified as a binding protein to the Src homology 2 domain of $p56^{lck}$ [6] and contains several functional domains such as zinc-finger motif, proline-rich regions and a PEST sequence. It is thought that the presence of p62 along with ubiquitin and other stress proteins of HSP 70 and HSP 25 in inclusions indicates a line of defense against misfolded proteins in chronic stress conditions [7,8].

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p62 is up-regulated in cultured neuronal cells during initiation of apoptosis and proteasomal inhibition, suggesting that this protein plays a protective role in pathological conditions [9]. p62 has been observed in ubiquitin-containing intraneuronal or intraglial inclusions such as neurofibrillary tangles [10], Pick bodies, Lewy bodies, and glial cytoplasmic inclusions in multiple system atrophy [11].

Recently, several investigators demonstrated that intracytoplasmic inclusions in the granule cells of dentate gyrus of patients with ALS-D contained both ubiquitin and p62 [12], although inclusions concerning spinal anterior horn cells were not examined. To better understand the protein aggregation containing ubiquitin in the anterior horn cells in ALS cases, we examined the spinal cords of 28 ALS cases including two ALS with dementia (ALS-D) and two basophilic inclusion type of ALS in addition to 11 non-ALS cases, using antibodies against p62 and ubiquitin. We found that p62 immunostainings with different types of reaction were observed in the spinal anterior horn cells and that these patterns could correspond to SLI, LBLI, and BI.

2. Materials and methods

We examined a total of 28 ALS lumber spinal cords samples (average age: 59.9 years old, sex: 12 males, 16 females), including two ALS-D cases (64-year-old female and 46-year-old male), and two ALS cases demonstrating basophilic inclusions (24- and 27-year-old females) in addition to those of 11 non-ALS cases including Alzheimer's disease, Creutzfeldt–Jakob disease, Parkinson's disease, cerebrovascular diseases, mitochondrial disease, and primary amyloidosis. Spinal cord tissues were all obtained from the institutes and universities. In all cases, the autopsies were performed in accordance with established procedures and the

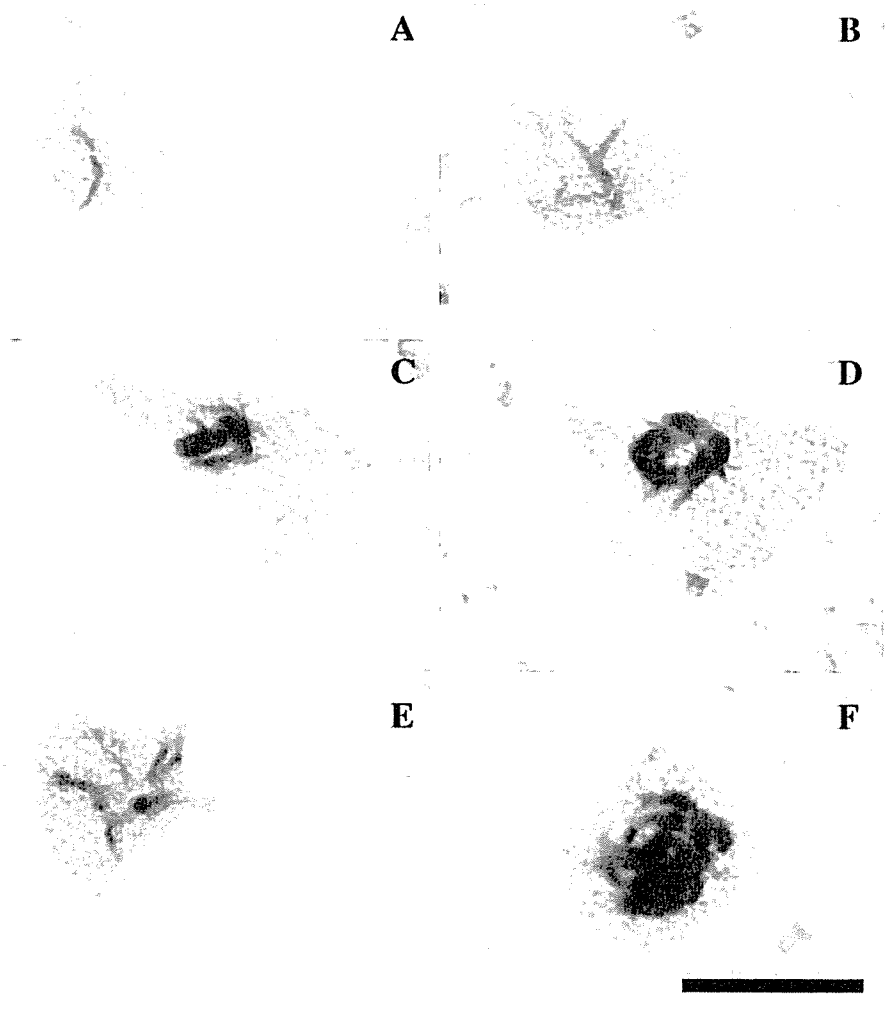

Fig. 1. p62 immunoreactivitiy for skein-like inclusions. A, C, E, F: Immunostaining for p62. B, D: Immunostaining for ubiquitin. Skein-like inclusions that were immunostained for ubiquitin (B and D) were p62-positive on serial sections (A and C). Other p62-positive skein-like inclusions are shown in E and F. Scale bar: 30 μm.