

## **Quantitative Highly-Sensitive Immunohistochemistry (Modified ImmunoMax) of HTLV-I p40tax and p27rex Proteins in HTLV-I-Associated Non-Neoplastic Lymphadenopathy (HANNLA) with Estimation of HTLV-I Dose by Polymerase Chain Reaction**

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Lymph nodes of HTLV-I-associated non-neoplastic lymphadenopathy (HANNLA) and adult T-cell leukemia/lymphoma (ATLL) were examined by means of quantitative highly sensitive immunohistochemistry (the modified ImmunoMax) of HTLV-I p40tax and p27rex proteins in order to see the relation of HTLV-I infection and the pathogenesis. Two cases of HANNLA showed follicular hyperplasia with follicle-lysis and paracortical hyperplasia. HTLV-I p40tax protein labeled by WATM-I and HTLV-I p27rex protein labeled by Rec-6 were seen dominantly in cytoplasm of paracortical lymphocytes in HANNLA and ATLL cells. In HANNLA cytoplasmic spotty stain of WATM-I was seen in dendritic cells of the paracortex. Image analysis of the modified ImmunoMax indicated gradual increase of p40tax from HANNLA to ATLL and appearance of the large amount of HTLV-I p27rex protein in the paracortical lymphocytes in HANNLA. Estimating dose of HTLV-I DNA forms by means of image analysis or polymerase chain reaction (PCR) for HTLV-I pX DNA and for human  $\beta$ -globin gene, increase of HTLV-I DNA forms in HANNLA suggested reproduction and reinfection of HTLV-I. Many LN-3 (Ia-like antigen)-positive dendritic cells in HANNLA suggested exogenous antigen-presenting. These findings suggested a possibility of an explosive increase of HTLV-I-infected T-cells in the paracortex of HANNLA through the host immunity. Follicle-lysis in HANNLA might be the expression of disordered B-cell differentiation in germinal centers under the regulatory T-cells loss caused by HTLV-1-induced apoptosis.

**Key words:** HTLV-I, p40tax, P27 rex, ImmunoMax, quantitative immunohistochemistry, image analysis, PCR, human  $\beta$ -globin gene, lymph node, ATLL, HTLV-1-associated non-neoplastic lymphadenopathy

### **Introduction**

Human T-cell leukemia virus type-1 (HTLV-1) causes neoplastic and non-neoplastic diseases in lymphoreticular tissue, skin, central nervous system and various organs (Takatsuki et al, 1992). Adult T-cell leukemia/lymphomas (ATLL) and HTLV-1-associated non-neoplastic lymphadenopathy (HANNLA) (Hasui et al. 1992, Ohshima et al. 1992) are the representative lymph node lesions related to HTLV-1 infection (Kikuchi et al. 1992).

HTLV-1 is a RNA retrovirus. Based on the molecular studies of HTLV-1 (Yoshida and Fujisawa. 1992) and on the life cycle of a retrovirus (Kobayashi. 1992), it was suggested that HTLV-1 has various forms in infected cells (Fig. 1). And its proviral DNA yields several proteins. Especially p40tax, one of the proteins of the HTLV-1 pX region, trans-activates expression of cellular genes and was expected to contribute to the oncogenesis of ATLL (Yoshida M and Fujisawa J. 1992). Recently, it was reported that p40tax binds competitively p16 and propels cell cycle of the cells (Suzuki et al. 1996). On the other hand, p40tax acts on its reproduction and p27rex induces HTLV-1 reproduction (Yoshida and Fujisawa. 1992). Therefore, immunohistochemical detection of p40tax and p27rex is expected to show the activation mode of HTLV-1 proviral DNA pX region and to predict HTLV-1 reproduction.

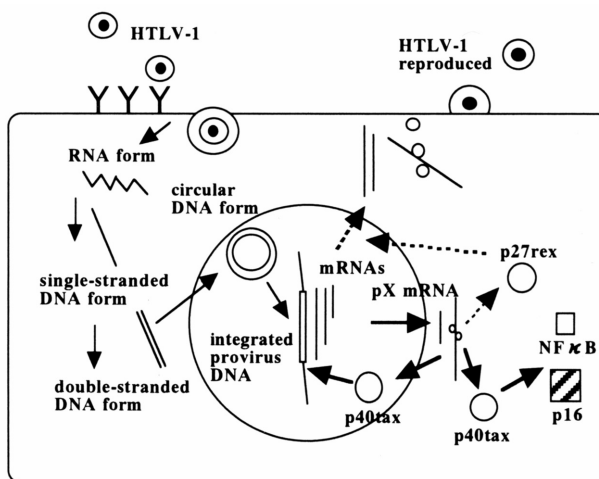
This study aimed to detect quantitatively p40tax and p27rex in the lymphocytes in HANNLA by means

of highly sensitive immunohistochemistry (Merz et al. 1995, Feller et al 1995, Sanno et al. 1996). The reproduction of HTLV-1 was estimated by means of polymerase chain reaction (PCR) for HTLV-1 pX region and image analysis of the agar-gel electrophoresis of the PCR products.

**FIGURE 1.** Life cycle of mLV-1 (Kobayashi. 1992, Yoshida and Fujisawa. 1992, Suzuki et al. 1996)

HTLV-1 is a RNA retrovirus. After infection, HTLV-1 RNA genome is transcribed by its reverse transcriptase to DNA genome. The DNA genome has several forms before its integration to host

cell DNA. From the integrated DNA at least 3 kinds of mRNAs are transcribed. HTLV-1 pX-mRNA produces p40tax, p27rex and p21. The p40tax activates its reproduction and trans-activates several host cell genes. On the other hand, the p40tax binds p16, NF  $\kappa$  B and the others in host cell cytoplasm and finally to contribute proliferation and oncogenesis of host cells. The p27rex modulates the activation of the proviral DNA to reproduce HTLV-1.



## Materials and Methods

Two cases of HANNLA were examined. Case 1 was 35 year old man. He complained swelling of left cervical lymph node and irregular tonsillar hyperplasia for several years without any other symptoms, physical and laboratory findings. Serologically anti-HTLV-1 antibodies were positive. His left cervical lymph node was removed and examined (HANNLA 1). Case 2 was 77 year old man complaining fever of unknown origin, leukocytosis and right inguinal lymph node swelling. His right inguinal lymph node was removed and examined (HANNLA 2). This case was reported as the case 3 in the previous paper (Hasui et al. 1992).

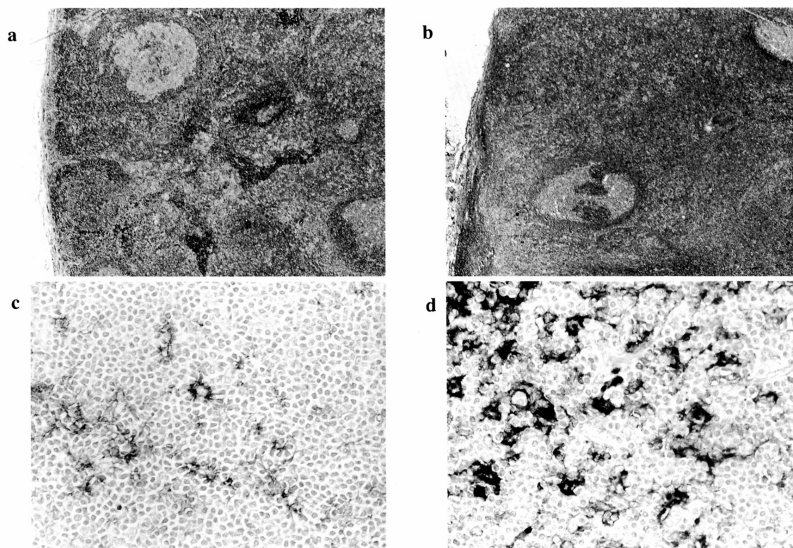
**FIGURE 2.** Histology of HTLV-1-associated non-neoplastic lymphadenopathy (HANNLA) and its paraffin-immunohistochemistry of LN-3 (Ia-like antigen, c and d).

A case (HANNLA 1) shows follicular hyperplasia and developing paracortex (a, c).

The other (HANNLA 2) shows developed paracortex, d).

Follicle-lysis are seen in the both cases (a, b).

In the paracortex of HANNLA LN-3-labeled Ia-like antigen-positive dendritic cells are seen (c, d).



One ATLL case was 77 year old man revealing systemic lymph node swelling. His cervical lymph node was examined and diagnosed histopathologically as T-cell pleomorphic lymphoma, large cell type according to the updated Kiel classification.

The lymph nodes of HANNLA 1 and 2 were examined by means of paraffin-immunohistochemistry (Hasui. 1991). The antibodies employed were MT-1, UCHL-1 and OPD4 for T cells, L-26, MB-1, LN-1

and LN-2 for B cells, anti-S100 protein antibody, anti-lysozyme antibody, Leu M1 and LN-3 (Ia-like antigen) for dendritic cells or histiocytes.

### **Highly sensitive immunohistochemistry of p40tax and p27rex and its quantitative evaluation**

Highly sensitive immunohistochemistry of HTLV-1 p40tax and p27rex was performed, introducing DAKO catalyzed signal amplification (CSA) system (DAKO, K1500) and endogenous biotin masking system (DAKO, XO590) to the capillary method of immunohistochemistry (MicroProbe™ staining system. Fisher Scientific) with modification according to ImmunoMax (Merz et al. 1995, Feller et al. 1995). The detail of the procedure (the modified ImmunoMax) is indicated in Table 1.

In this study, antigen-retrieval pretreatment of the dewaxed section was performed by incubating and heating sections in 4M urea solution for 5 min. after boiling in a high pressure cookpot and by cooling sections rapidly in several times rinse in pure water (Hasui et al. 1995). After destroying endogenous peroxidase activity, the sections were setted in the slide holder of MicroProbe. The following processes of the modified ImmunoMax were performed by means of MicroProbe.

Monoclonal antibodies (MoAb) employed were mouse MoAb Lt-4 and rat MoAbs WATM-1 and WATM-3 against p40tax (TAX1) (Tanaka et al. 1992) and rat MoAb Rec-6 for p27rex. Rec-6 is a rat monoclonal IgG antibody specific for a oligopeptides corresponding to a C-terminal region of p27rex

(SGEAPLSACTSTSFPPSPGPSCPT) (Tanaka et al. unpublished). The detail character of the Rec-6 will be described elsewhere. After blocking non-specific binding of the primary antibody to proteins, the primary antibody reaction was done for 1 hour at the room temperature. The washing after the reaction comprised more than 3 cycles of rinse in the preheated 0.1% Triton X100 Tris buffer and hold for 1 min. at 35 to 40°C.

Endogenous biotin masking was done after the primary antibody reaction, reducing an effect of residual H<sub>2</sub>O<sub>2</sub> to dissociate biotin-avidin binding (Tsutsumi Y. 1985).

The following processes were performed according to Table 1 and the reactions were performed at the room temperature.

Control negative stain was prepared in the performance of the modified ImmunoMax, employing the primary antibody dilution solution without the primary antibody.

**Table 1.** Procedure of the modified ImmunoMax

1, Dewax
2, Antigen retrieval
3, Destroying endogenous peroxidase activity
a) Incubate in 3% H <sub>2</sub> O <sub>2</sub> PBS for 20 min. at room temperature
b) Wash 3 times by water and Wash(#1)
4, Blocking non-specific binding of primary antibody to proteins
a) Incubate in 2% casein PBS including horse serum (80μl for 1 ml) for mouse primary antibody for 30 min. at room temperature(%1)
b) Remove excess the solution and no wash
5, Primary antibody reaction
Dilute primary antibody in BSA-PBS including rabbit serum (7:1), incubate(&1 or &2) and Wash(#1)
6, Endogenous biotin masking (DAKO Endogenous Biotin Blocking Kit)
a) Incubate in 0.1% avidin Tris including 0.03M NaCl for 15 min. at room temperature(%1) and Wash(#1)
b) Incubate in 0.01% biotin Tris including 0.03M NaCl for 15 min. at room temperature(%1) and Wash(#1)
7, Secondary antibody reaction for 30 min. to 1 hour at room temperature(%2) and Wash(#1)
8, Streptavidin-peroxidase complex reaction for 15 min. at room temperature(%2) and Wash(#1)
9, Catalyze reaction of biotinylated tyramide for 15 min. at room temperature and Wash(#2)
10, Streptavidin-peroxidase complex reaction for 15 min. at room temperature(%2) and Wash(#2)
11, Development of color reaction (DAB-H <sub>2</sub> O <sub>2</sub> peroxidase reaction for 5 min. at room temperature) and wash in water several times
12, Nuclear counter stain by methyl-green
13, Dehydration and mount in plastic medium
.....
Quantitative procedure should be performed by capillary method such as MicroProbe
In the routine procedure, demarcate the area should be stained by DAKO pen in order to avoid dryness of the section in the reaction between wash by water and Wash(#1) by Tris in the destroying endogenous peroxidase activity.
Tris: 0.1% Tween 20 or 0.1% Triton X100 Tris
Primary antibody reaction:
For routine procedure,
Incubate&1: Incubate sections overnight at 4°C
For quantitative procedure by MicroProbe,
Incubate&2: Incubate sections for 30 min. or 1 hour at 60°C or at the room temperature
Wash:
For routine procedure,
Wash(#1): Wash in Tris for 5 min. 3 times at 35~40°C
Wash(#2): Wash in Tris for 5 min. 3 times at room temperature
For quantitative procedure,
Wash(#1): More than 3 cycles of rinse in Tris 3 times and hold in Tris for 1 min. at 40 or 60°C
Wash(#2): More than 3 cycles of rinse in Tris 3 times and hold in Tris for 1 min. at room temperature
For quantitative procedure
%1: For 10 min. at 60°C or as indicated.
%2: For 5 min. at 60°C or as indicated.

Quantitative evaluation of the immunohistochemistry was made on the color slide images of the stain (Goto et al. 1992). Color slide photos were taken at the magnification of 40x1.25 under auto-focusing and -exposing functions by Nikon Microphoto-FXA. An unit area of color slide photo image was translated as a computer color image by a slide scanner (Polascan 35, Polaroid). The color images were divided into red, green and blue (RGB) images by the channel division function of Adobe Photoshop. Nuclei were counted on the red (R) image. On the blue (B) image brown products of DAB-peroxidase reaction were estimated in area and mean density by an image analysis program, NIH-Image. The product of the area and the mean density was treated as the amount of antigens labeled.

**Estimation of HTLV-I dose by means of image analysis of agar-gel electrophoresis of PCR product**

DNA material for the PCR was extracted from the paraffin-embedded tissue sections of the two cases of HANNLA, the one case of ATLL and MT-2 cell block (Supplied from Miyoshi I, Kochi Medical University to Sonoda S, Kagoshima University). Dewaxed section fragments were treated by proteinase K at 37°C for 2 days. Destroying activity of the proteinase K by incubating the microtubes in boiling water for 3 min, 5 μl of the extracted DNA material was applied to the PCR for HTLV-1 pX region and for human β-globin (HBG) gene.

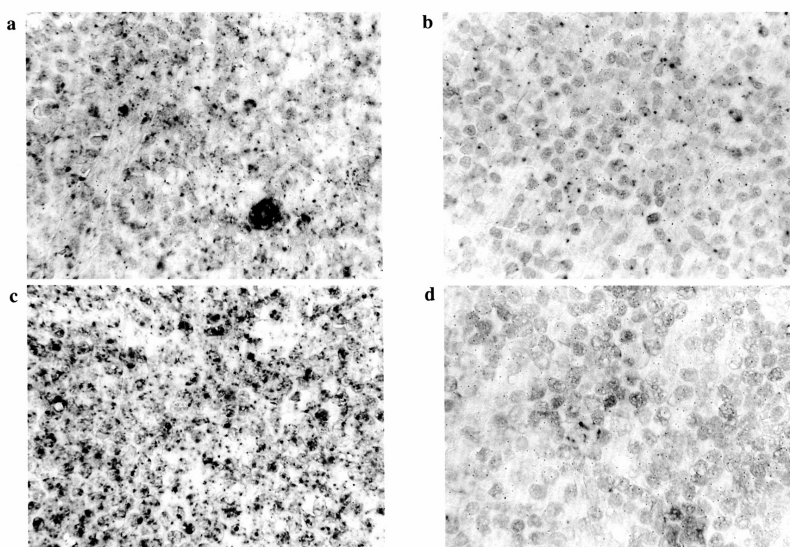
Employing a primer pair SK43-44 for HTLV-1 pX region or a primer pair GH20-21 for HBG gene, PCR was performed in the following protocol comprising pre-denature at 94°C for 5 min., 30 cycles of denature at 94°C for 30 sec., annealing at 45°C for SK43-44 or at 55°C for GH20-21 for 30 sec., extension at 72°C for 30 sec. and after-extension at 72°C for 5 min. Second PCR was performed in the same protocol, applying 5 μl of the 1,000 times diluted product of the first PCR as the template.

Expected lengths of DNA amplified were 159 bp in the PCR of HTLV-1 pX region and 408 bp and 244 bp in the PCR of HBG gene. Twenty μl of the PCR product was loaded in 4% agar-gel electrophoresis, which was stained by ethidium bromide. Polaroid photo of the electrophoresis was transferred to a computer image by means of an image scanner (EPSON, GT-6000). In the computer image of the agar-gel electrophoresis ethidium bromide fluorescent quantitation (Sambrook J et al. 1989) was performed by using gel-plot macro function of NIH-Image. Each estimated amount of DNA in the bands at the expected length was standardized by that of the band at 200 bp of the molecular length marker (Marker 9, φ174/Hinf I digested, Nippon Gene Co.).

Dose of HTLV-1 DNA forms was calculated as a ratio of the DNA amplified in the PCR for HTLV-1 pX region to that of the PCR of HBG gene. When the ratio in MT-2 was 7 (Seven copies of a pair of integrated proviral DNA. Yashiki, unpublished) and was 1 in the case of ATLL, it was estimated how many copies of HTLV-1's DNA forms the two cases of HANNLA counted.

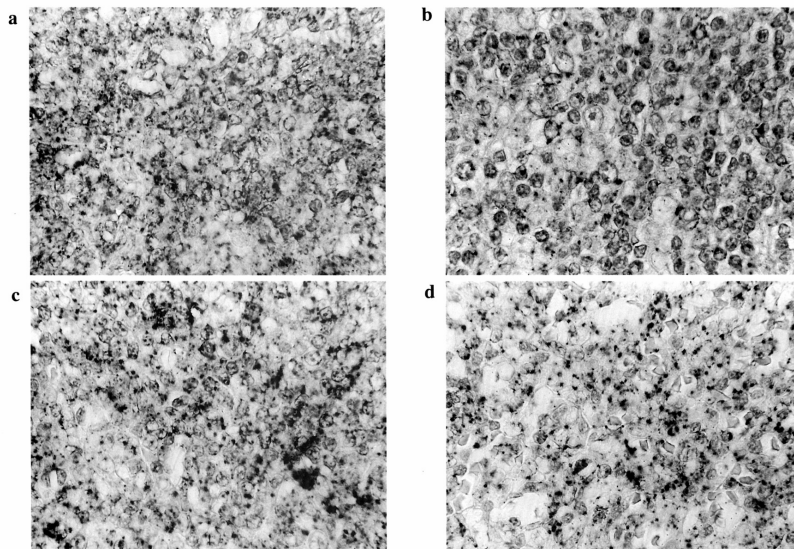
**FIGURE 3.** The modified ImmunoMax of Lt-4 (a), WATM-1 (b), WATM-3 (c) and Rec-6 (d) in tLANNLA 1.

In the developing paracortex lymphocytes are labeled by the MoAbs: Lt-4, WATM-1 and WATM-3, against p40tax. A cell revealing strong cytoplasmic reaction may be mast cell in the Lt-4 stain (a). Clusters of granular reaction products are seen in some dendritic cells in the Lt-4 and WATM-1 stains (a, b). In this area only a few cells are positive for Rec-6 against p27rex (d).



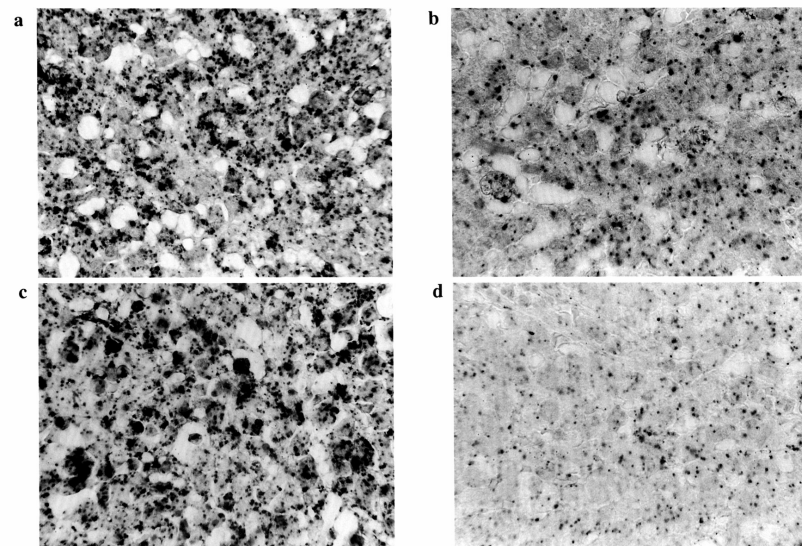
**FIGURE 4.** The modified ImmunoMax of Lt-4 (a), WATM-1 (b), WATM-3 (c) and Rec-6 (d) in HAN-NLA2.

Many lymphocytes in the developed paracortex are labeled by the three MoAbs: Lt-4, WATM-1 and WATM-3, against p40tax in different positive figures in each stain (a, b, c). Many lymphocytes are also labeled by Rec-6 against p27rex (d).



**FIGURE 5.** The modified ImmunoMax of Lt-4 (a), WATM-1 (b), WATM-3 (c) and Rec-6 (d) in ATLL.

Many ATLL cells are labeled by the MoAbs: Lt-4, WATM-1 and WATM-3, against p40tax in different pattern in each stain (a, b, c). In each figure some nuclei show Positive stain. Many ATLL cells are also labeled by Rec-6 against p27 rex (d).



## Results

The two lymph nodes of HANNLA show follicular hyperplasia, follicle-lysis and paracortical hyperplasia (Fig.2a and b). Follicular hyperplasia is dominant in HANNLA 1. Paracortical hyperplasia is marked in HANNLA 2. In the both lymph nodes some of germinal centers reveal follicle-lysis with infiltration of many T-cells labeled by OPD4 or UCHL-1. In the areas of paracortical hyperplasia no obvious immunoblasts are seen. Many S100- or LN-3 (Ia-like antigen)-positive dendritic cells are seen (Fig. 2c and d).

In HANNLA 1, the MoAbs reacted lymphocytes and dendritic cells in the developing paracortex. WATM-3 yielded the strongest reaction product in cytoplasm and in some nuclei (Fig. 3c). Lt-4 (Fig. 3a) and WATM-1 (Fig. 3b) showed granular or spotty stain in cytoplasm of lymphocytes. Some dendritic cells showed clusters of granular reaction products of Lt-4 or WATM-1 in their cytoplasm (Fig. 3a and b). Distribution of Rec-6 positive cells varies area to area in the paracortex. In an area (Fig. 3d) granular or spotty and weak stain of Rec-6 is seen only in cytoplasm of some lymphocytes and dendritic cells.

In HANNLA 2, the lymphocytes in the developed paracortex showed granular reactions in the all stains

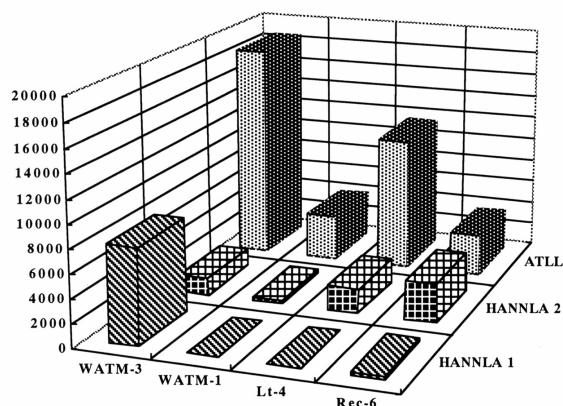
of Lt-4, WATM-1, WATM-3 and Rec-6 (Fig. 4). WATM-3 (Fig.4c) yielded spotty stain on some nuclei. In HANNLA 1 and 2 and ATLL the stain of Rex-6 was the strongest in HANNLA 2 (Fig. 4d).

In ATLL the MoAbs against p40tax reacted lymphoma cells strongly in the order of WATM-3, Lt-4 and WATM-1 (Fig. 5). WATM-3 showed spotty stain in several nuclei (Fig. 5c).

**FIGURE 6.** Quantitative estimation of the modified ImmunoMax of the MoAbs against p40tax and p27rex. Value is product of the area and the mean density per one cell per a unit area according to an arbitrary unit.

In the MoAbs against p40tax, antigens labeled by Lt-4 and WATM-1 increased gradually in the order of HANNLA 1, HANNLA 2 and ATLL. The amount of the antigen labeled by WATM-1 is the smallest. The amount of the antigen labeled by Rec-6 was large in HANNLA 2 and ATLL.

In HANNLA 1, 260 to 296 cells were counted in the unit area of the paracortex. There were 204 to 249 cells in HANNLA 2 and 107 to 141 cells in ATLL.

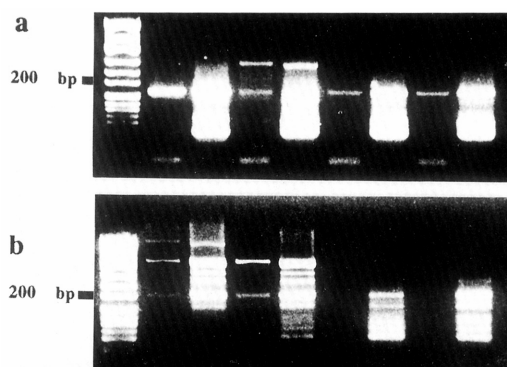


**FIGURE 7.** Agar-gel electrophoresis of PCR

a) PCR for HTLV-1 pX Tax region. The expected length of the amplified DNA is 159bp. A band of the amplified DNA is seen at the expected length in each of the 1st PCR products. Only in the 1st PCR of HANNLA 1 a band of the concatamer is seen at 318bp.

b) PCR for human  $\beta$  globin gene. The expected length of the amplified DNA is 408bp. But in the 1st PCR of MT-2 cell line and HANNLA 1, bands of the amplified DNA are seen at 408bp and at 244bp. The band at 244 bp is also specific in this PCR (Informed from TAKARA Technical Support Line).

In a) and b) from the left side,  
 the first lane the molecular length marker, Marker 9  
 the second lane the 1st PCR product of MT-2 cell line  
 the third lane the 2nd PCR product of MT-2 cell line  
 the fourth lane the 1st PCR product of HANNLA 1  
 the fifth lane the 2nd PCR product of HANNLA 1  
 the sixth lane the 1st PCR products of HANNLA 2



the seventh lane the 2nd PCR product of HANNLA 2  
 the eighth lane the 1st PCR product of ATLL  
 the ninth lane the 2nd PCR product of ATLL

Figure 6 indicated graphically the estimated volumes of the antigens labeled by the MoAbs. Estimated volumes of p40tax-antigens labeled by Lt-4 and WATM-1 increased gradually in the order of HANNLA 1, HANNLA 2 and ATLL, when that of WATM-3 was larger in HANNLA 1 than HANNLA 2. A large amount of p27rex-antigen labeled by Rec-6 was seen in HANNLA 2 and in ATLL. Nuclei decreased in a unit area gradually in the order of HANNLA 1, HANNLA 2 and ATLL.

Figure 7 showed agar-gel electrophoresis of the first and second PCR of HTLV-1 pX region and HBG gene. In the first PCR of HTLV-1 pX region a band of amplified DNA was seen at the expected length 159 bp in the lanes of MT-2, 2 cases of HANNLA and ATLL. In the lane of HANNLA 1 the other band of the concatamer DNA was found about at 311 bp. In the PCR of HBG gene a band of DNA amplified was seen in the all four lanes of the second PCR at 244 bp. The band at 408 bp was seen only in the lanes of MT-2 and HANNLA 1 in the first PCR and was quite faint in HANNLA2 in the second PCR.

Ethidium bromide fluorescent quantitation of double-stranded DNA in the bands in the agar-gel electrophoresis indicated that there were 1.6 copies/cell of HTLV-1 DNA forms in HANNLA1 and 5.5 copies/cell in HANNLA 2.

## Discussion

HANNLA was reported (Hasui et al. 1992) to show follicle-lysis, which is often found in persistent generalized lymphadenopathy in human immunodeficiency virus type-1 infection, in HTLV-1 carriers. The same kind of lymph node lesion was reported as HTLV-1-associated lymphadenitis by Ohshima et al (1992) to show follicular hyperplasia with and without dermatopathic lymphadenopathy (DPLA)-like paracortical enlargement. The two cases of HANNLA in this study have the both features. HANNLA and HTLV-1-associated lymphadenitis mean the same lymph node lesion in HTLV-1 carriers.

It could be estimated in the stain of serially 10 times diluted primary antibody (Lt-4 or NCL-CD5) solutions (1:100 to 1:1,000,000,000) on MT-2 cell block sections or human tonsillar tissue that the highly sensitive immunohistochemistry, such as ImmunoMax (Merz. 1995, Feller. 1995) and DAKO CSA, is 1,000 times more sensitive than Elite ABC (Hasui. unpublished). Elite ABC is the same in sensitivity to streptavidin-biotin complex method (sABC). The both methods were reported to be more than 4 times more sensitive than ABC, although the end point of the stain was not reported in the primary antibody-dilution experiments (Happerfield. 1993). But in the end point of the reaction Elite ABC was 10 to 100 times more sensitive than ABC in the above mentioned serially dilution of the primary antibodies (Hasui. unpublished). On the other hand, it was reported (Sanno. 1996) that DAKO CSA was about 1,000 times sensitive than indirect immunohistochemistry and 100 times sensitive than ABC and was free from endogenous biotin reaction. Our performance of DAKO CSA showed endogenous biotin reaction in hepatic cells, gastric glands, skin appendage glands and tonsillar squamous epithelium. Endogenous biotin reaction in gastric glands was recognized also in the performance of DAKO CSA in Technical Division of DAKO Japan. The performance of DAKO CSA by Sanno et al (1996) may be less sensitive than our performance. The modified ImmunoMax needed blocking of non-specific binding of the primary and secondary antibodies, masking of endogenous biotin and complete post-reaction wash in the pre-heated Tris buffer including surfactant.

The modified ImmunoMax was performed in this study by means of capillary method (MicroProbe<sup>TM</sup>, Fisher Scientific), controlling reaction time and temperature. Because the routine procedure of the modified ImmunoMax treating 20 sections needs longer time in each process in accordance with the order of the sections from the first to the 20th, quantitative evaluation of the reaction needed the capillary method.

This study employed three MoAbs against HTLV-1 p40tax. Because there was difference in their stains in HANNLA and ATLL (Fig. 3, 4 and 5), it must be discussed which of the three MoAbs labels antigen specific for HTLV-1 p40tax in vivo. WATM-1 detected the antigen the most specific for HTLV-1 p40tax, because the estimated amount of antigen labeled by WATM-1 was the smallest (Fig. 6). It was also suggested in comparison of the stains of Lt-4 and WATM-1 in T-cell malignant lymphomas (Hasui, unpublished). On the other hand, there is a possibility that HTLV-1 p40tax changes its antigen profile in nucleus and in cytoplasm. The large amount of the antigen labeled by WATM-3 in HANNLA 1 (Fig. 6) suggested the possibility, because WATM-3 reacts a part of Tax 1 peptide different from the part reacted by Lt-4 and WATM-1 (Tanaka. 1992). But WATM-3 was also reported to show a cross reaction (Tanaka. 1992). The large amount of the antigen labeled by WATM-3 in HANNLA 1 may be the product of the cross reaction of WATM-3.

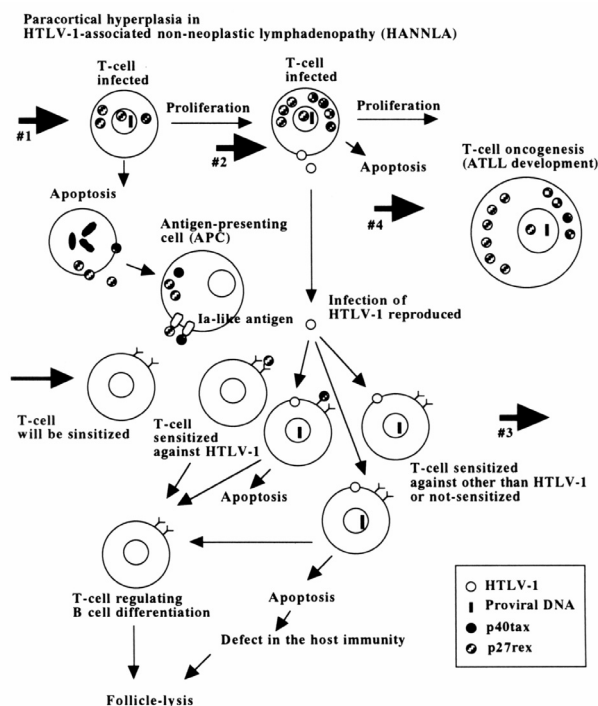
This study employed only one MoAb Rex-6 for detecting HTLV-1 p27rex. It was unknown in this study whether the antigen labeled by Rec-6 was specific for HTLV-1 p27rex in vivo. Because HTLV-1 p27rex induces reproduction of HTLV-1, this study tried to see whether HTLV-1 DNA forms which can be detected by the PCR increase in HANNLA 2 revealing the highest estimated amount of the antigen labeled by Rec-6. HTLV-1 itself can not survive so long time in the cell free condition. HTLV-1 reproduced newly must reinfect cells. The reinfected HTLV-1 takes several DNA forms before its integration to the host cell DNA (Fig. 1). Thus, the dose of HTLV-1 reproduced could be evaluated by PCR. Comparing the amplification of HBG gene by PCR, semiquantitative evaluation of HTLV-1 dose can be made (Sueyoshi. 1996). This study tried to measure ethidium bromide fluorescence of the bands in agar-gel electrophoresis of the PCR products, although an exponential increase of the amplified DNA and the difference in the annealing affinity of primers would modify the amount of the PCR products.

The gradual increase in the amount of HTLV-1 p40tax from HANNLA to ATLL suggested contribution of the p40tax on the oncogenesis of ATLL (Yoshida and Fujisawa. 1992). The appearance of a large amount of p27rex was shown in paracortical hyperplasia in HANNLA in this study. The estimated copies

of HTLV-1 DNA forms, 5.5 copies/cell, in HANNLA 2 suggested reproduction of HTLV-1 and reinfection of the HTLV-1 reproduced. HTLV-1 p40tax-induced apoptosis (Yamada et al. 1994) may contribute the decrease of cells in a unit area in the order of HANNLA 1, HANNLA 2 and ATLL.

FIGURE 8. HTLV-1 infection and pathogenesis of tHTLV-1-associated non-neoplastic lymphadenopathy (HANNLA)

T cell infected by HTLV-1 proliferates or goes to apoptosis. HTLV-1 p40tax, p27rex and the other HTLV-1-related antigens coming from the apoptotic T-cell are caught by dendritic cell. The dendritic cell grows to an antigen-presenting cell (APC) presenting the HTLV-1-related antigens with Ia-like antigen (HLA-DR, class II molecule). T cell is sensitized by the APC and modulate the host immunity against HTLV-1. On the other hand, some of T cells infected by HTLV-1 yield a large amount of p27rex and produce HTLV-1 under the modulation of the p27rex. The HTLV-1 reproduced infects T cells and the other cells in the neighborhood. The T cells infected by the HTLV-1 reproduced are sensitized against HTLV-1 or the other antigens, or are not sensitized. These infected T cells undergo the pathway from the arrow #3 to the arrow #1, #2 or #4. The circuit from the arrow #3 to the arrow #1 would make an exponent increase of HTLV-1-infected T cells in a host through recirculation of T-cells. Apoptosis of sensitized T cells would induce defects in the host immunity. Some of T cells newly sensitized may enter into germinal centers to regulate B-cell differentiation to improve the defects. Such germinal centers would manifest follicle-lysis. It is unknown which of T cells in this schema develop to ATLL cells (the arrow #4). But at least T-cells getting a large amount of p40tax would onco-develop to be ATLL cells.



The relation between HTLV-1 infection and pathogenesis of HANNLA is explained in Fig. 8. As seen in Fig. 3, in the early development of the paracortex in HANNLA a small amount of p40tax would induce proliferation of T cells and apoptosis of some of them. Clusters of p40tax in dendritic cells suggested that the p40tax from the apoptotic cells must be phagocytized by the dendritic cells. These dendritic cells had LN-3-labeled Ia-like antigen (HLA-DR), which appears in a process to present exogenous antigen on cell surface. Probably immunity against p40tax and other proteins related HTLV-1 would occur, depending on the antigen-presentation of these dendritic cells. In the developed paracortex in HANNLA the increased p27rex (Fig. 4d) induced reproduction of HTLV-1. Newly reproduced HTLV-1 infects pre-existing lymphocytes and the lymphocytes entering into the paracortex to be sensitized. The HTLV-1 infection in the paracortex would induce explosive expansion of HTLV-1-infected T-cells through recirculation of the HTLV-1 infected T-cells to general body. On the other hand, a combination of the T-cells sensitized against HTLV-1 and a loss of pre-existing memory cells may induce follicular hyperplasia and follicle-lysis with infiltration of many T-cells infected or not-infected by HTLV-1.

It is unknown where the first T-cell with HTLV-1 infection appears in the arrow #1 pathway from. HTLV-1 must be survive latently in some cells after perinatal infection. Epithelioid cells, dendritic cells and histiocytes might be the candidates, because the cells in some low grade T-cell lymphomas were positive in the in-situ-hybridization for HTLV-1 pX tax mRNA (Hasui and Sato. 1994).

We also recognized p40tax and p27rex in germinal centers of HANNLA. Further studies are necessary to understand whether these HTLV-1-related proteins in germinal center reflect processes of B-cell immunity against HTLV-1 or HTLV-1 infection in germinal center cells.

The authors believe a possibility that the suppression of synthesis of HTLV-1-related proteins in the



paracortical lymphocytes in HANNLA stops expansion of HTLV-1 infection and finally ATLL development in a HTLV-1-carrier.

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