Human T-cell leukemia virus type 1 (HTLV-1) is an etiologic agent of adult T-cell leukemia (ATL) and is also associated with a chronic myelopathy named HTLV-1-associated myelopathy (HAM) or tropical spastic paraparesis (TSP). Proviruses of HTLV-1 have been reported to be clonally integrated in ATL patients, but randomly integrated in HAM/TSP patients. Furthermore, the replication of HTLV-1 was found to be more efficient in HAM/TSP patients than in general carriers. Thus, the mechanism of efficient HTLV-1 replication and clonal selection of HTLV-1-infected cells in these diseases are of interest.

A regulatory gene tax, which is located at the pX region of the HTLV-1 genome, was identified as the gene responsible for viral replication, and the abnormal growth and immortalization of HTLV-1-infected T cells. The Tax protein transactivates the expression not only of the viral gene but also of cellular genes, including the interleukin-2 receptor α (IL-2Ra) and the proto-oncogene (c-fos) and some others. In fact, expression of the genes in the HTLV-1 pX region was shown to immortalize normal T cells in an IL-2-dependent fashion. However, HTLV-1-infected cells in asymptomatic carriers should proliferate more efficiently than the other population without malignant transformation. The results showed that one fifth of the HAM/TSP patients and of their seropositive family members harbored subpopulation(s) of clonally proliferated cells infected with HTLV-1, although they still maintained randomly infected cells as a major population. These clones were stable during examination periods of 4 months to 3 years. However, these carriers or HAM/TSP patients did not show any significant indication of ATL. This extremely high frequency of clonal expansion of HTLV-1-infected cells indicates that some clones of HTLV-1-infected cells have a tendency to proliferate more efficiently than the other population without malignant transformation.

**RESULTS**

Diagnosis. Most HAM/TSP patients surveyed in this study were residents of Kagoshima, Kyushu, Japan, where HTLV-1 is endemic. The clinical diagnosis of HAM/TSP was based on the criteria proposed by Osame et al.

**Blot analysis of HTLV-1 DNA.** Total cellular DNA was extracted from peripheral blood lymphocytes that had been frozen soon after their isolation. The DNA (10 μg) was digested with Pst I and subjected to Southern blot hybridization as described previously. The DNA was transferred to nylon filters and hybridized with 32P-labeled HTLV-1 DNA containing the whole viral sequence (Fig 1A). When DNA bands derived from the internal regions of the HTLV-1 proviral genome were detected after exposure for 7 days (Fig 1B), the sample was scored as positive for a detectable level of HTLV-1 DNA. In this assay, infection into 2% to 3% of the cells in the tested sample was detectable. The same filters were rehybridized with the 32P-labeled HTLV-1 long terminal repeat (LTR) probe (Fig 1A). When at least one discrete band was detected after exposure for 7 days, the sample was judged to contain clonally proliferated cells infected with HTLV-1 (Fig 1B). Unclear results were scored as negative.
Fig 1. (A) Schematic illustration of the HTLV-1 genome, restriction map and probes. (■) The Pst I site; (□) the regions of the probe used. (B) DNA blot analysis of the HTLV-1 proviral genome in peripheral blood lymphocytes of HAM/TSP patients (A through C and F through I) and carriers in HAM/TSP families (D and E). Ten micrograms of cellular DNA was digested with Pst I and subjected to the standard Southern blot procedure. The filter was hybridized with a total HTLV-1 probe (lane 1) and then with an LTR probe (lane 2). Cases A through G showed the viral-cellular junction bands with LTR probe (marked with arrowheads), in addition to three strong internal bands (A through E) or two internal bands (F and G). Cases H and I showed polyclonal integration.

digestion. Pst I cleaves the HTLV-1 provirus at several sites and produces three large fragments from the internal region and two from the viral-cellular junctions, if the provirus is intact. The probe used in the first survey covered the whole sequence of the HTLV-1 genome as described previously.

Of 99 HAM/TSP patients who were diagnosed as having typical HAM/TSP, 84 patients (84%) showed two or three visible bands that were derived from the internal part of the integrated proviral genomes (Fig 1). The results confirmed our previous observation that HTLV-1 replicated efficiently in HAM/TSP patients. To understand whether this efficient replication of HTLV-1 was specific to the patients, we also surveyed asymptomatic carriers in families of the HAM/TSP patients. Of the 50 subjects examined, 26 (52%) showed specific bands of HTLV-1. This frequency in HAM/TSP families was significantly higher than that (20%) among general asymptomatic carriers, which was not specifically classified (Table 1). Therefore, the efficient replication of HTLV-1 was observed not only in patients with HAM/TSP, but also in family members of HAM/TSP patients. We could not detect any differences between the

<p>| Table 1. Detection and Clonality of the Integrated HTLV-1 Proviral DNA in Peripheral Lymphocytes of HAM/TSP Patients and HTLV-1 Carriers |
|---------------------------------|---|---|---|
| Diagnosis | HAM | HAM/TSP | Non-HAM |</p>
<table>
<thead>
<tr>
<th>Family Carrier</th>
<th>Total Cases</th>
<th>HTLV-1 DNA*</th>
<th>% Incidence</th>
<th>Clonality of Integration†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAM/TSP</td>
<td>99</td>
<td>+</td>
<td>84</td>
<td>M</td>
</tr>
<tr>
<td>Family Carrier</td>
<td>50</td>
<td>+</td>
<td>84</td>
<td>R</td>
</tr>
<tr>
<td>Non-HAM Carrier</td>
<td>60</td>
<td>-</td>
<td>19</td>
<td>M</td>
</tr>
<tr>
<td>Cases</td>
<td>84</td>
<td>15</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>% Incidence</td>
<td>84</td>
<td>15</td>
<td>52</td>
<td>20</td>
</tr>
<tr>
<td>Clonality of Integration†</td>
<td>M</td>
<td>R</td>
<td>M</td>
<td>R</td>
</tr>
<tr>
<td>Cases</td>
<td>19</td>
<td>65</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>% Incidence</td>
<td>19</td>
<td>65</td>
<td>16</td>
<td>36</td>
</tr>
</tbody>
</table>

*Samples that gave visible bands corresponding to viral internal fragments after 7 days of exposure were scored as (+), and others as (-).
†Samples that gave discrete bands with the LTR band after 7 days of exposure were scored as clonal integration (M) and others as random integration (R).
clinical features of HAM/TSP patients with and without a detectable level of HTLV-1 DNA.

Survey of clonality of HTLV-1-infected cells. Previously, we have reported concurrent infection with HAM/TSP and ATL in three of 155 (2%) HAM/TSP patients. To test the clonality of the proviral integration sites, the HTLV-1 LTR was used as a probe (Fig 1A). The LTR probe detects only fragments from both termini of proviruses that contain the cellular-viral junction sequence, and theoretically, it should detect two bands for each provirus integrated into cellular DNA. However, even cases with a single band were judged to have clonally proliferated infected cells, because the two fragments could overlap each other on gel electrophoresis or the provirus could be defective.

Using the LTR as a probe, 19 of 99 (19%) HAM/TSP patients examined showed at least one discrete band of HTLV-1 proviral DNA. Some typical results are shown in Fig 1. These observations indicate that 19% of the HAM/TSP patients harbored clonally proliferated cells that had been infected. The bands detected with the LTR probe were more faint than those of the internal fragments, indicating that the population of clonally expanded cells were not major among infected cells. These observations also excluded a possibility that an extra band with the LTR sequence could be derived from a mutation forming a new *Pst* I site in the LTR, because the additional bands should be in the same densities as those of the internal bands if it was caused by such mutation.

These patients carrying clonally expanded cells did not show any sign of ATL. Furthermore, careful comparison of these two HAM/TSP groups, with or without clonal proliferation of HTLV-1-infected cells, indicated no significant differences in their clinical features including age of onset, progression rate of disease, or history of blood transfusion.

In a survey with the LTR probe, of 50 asymptomatic carriers in families of HAM/TSP patients, eight cases (16%) gave more than one band. This frequency was comparable with that among HAM/TSP patients. In this survey, no asymptomatic carriers in the general population were found to have clonal proliferation of infected cells (Table 1). However, the number of carriers was too small to reach a definite conclusion. In fact, Ikeda et al recently found that about 2% of general carriers harbored clonally expanded cells.

During these surveys we made two exceptional findings. First, three of the 19 HAM cases with clonal expansion gave more than two bands with the LTR probe (examples in Fig 1C and E), indicating either two infected cell clones, each carrying one copy of the provirus, or one cell clone carrying two copies of the provirus. These two possibilities could not be easily distinguished. Second, three cases gave only two internal fragments (examples in Fig 1F and G), whereas the other 16 cases gave three internal fragments as expected. These three abnormal cases could be attributable to a small change in the HTLV-1 genome, such as a point mutation or a small deletion at one of the *Pst* I sites. Because the other bands were of normal size, large or complicated alterations should not be possible. In addition, if these represent a large deletion in the genome, the defective provirus would be defective in replication, yet the patients had a large population of randomly integrated proviruses, indicating that the variant viruses replicate actively.

Stability of the infected cell clone. We examined the stability of clonally proliferated cells by comparing cell clones obtained from individual patients on different occasions. Three HAM/TSP patients maintained the same clone after 4, 6, and 7 months, respectively. Two asymptomatic carriers also showed the same clone after 4 months and 3 years (Fig 2). Therefore, the clonal proliferation of HTLV-1-infected cells found in these HAM/TSP patients and carriers was not transient during a short period, but the selected clones were stable. However, we cannot determine whether the levels of the clonally expanded cells were constant, because the samples were analyzed in separate experiments. No appearance or disappearance of clonally expanded cells was observed.

DISCUSSION

In this study, we detected clonal proliferation of HTLV-1-infected cells in 19% of the HAM/TSP patients and 16% of the carriers in families of the HAM/TSP patients examined. These frequencies correspond to 26% and 32% among patients or carriers who had detectable levels of HTLV-1 proviruses, respectively. Retroviruses integrate their proviral DNA into nonspecific sites in the host chromosomal DNA. Therefore, detectable level of a single integration site reflects clonal proliferation of infected cells. This frequency of clonal proliferation of infected cells of 19% in HAM/TSP patients and their family members was unexpectedly high, because we previously reported random integration in HAM/TSP patients. Furthermore, none of these cases showed any signs of ATL, and the infected cells clonally proliferated in these HAM/TSP patients and their family members were stable for examination periods of up to 3 years.

Increase of randomly infected cells is the result of either or both HTLV-1 replication and proliferation of randomly infected cells. Proliferation of infected cells could be stimulated by the viral Tax protein through transactivation of gene expressions of IL-2Ra and c-fos. As the Tax protein was not expressed constitutively at a significant level in vivo, it was suggested that random cell proliferation stimulated by the Tax protein would occur transiently during transient expression of the viral proteins. The observed higher densities of HTLV-1-infected cells in patients with HAM/TSP and their family members could be accomplished through either, or both mechanisms and could be associated with some host genetic factors. The host genetic factors could be the HLA haplotypes as proposed for high immunologic response to HTLV-1 infection. Of these randomly infected cells, a cell clone is selected for preferential growth and triggers ATL development. Previously, we postulated a rare genetic alteration in the process of clonal selection during leukemogenesis of ATL. However, the high frequency of clonal proliferation of infected cells in HAM/TSP patients and their family members may suggest rather epigenetic factors or an unexpectedly highly frequent mutation. We cannot distin-
guish these possibilities, although it is possible that HTLV-1 infects a particular population of T cells in which Tax is more efficiently expressed. Another possibility is infection of T cells that recognize HTLV-1 antigens, and the infected cells are therefore continuously stimulated by the viral antigens supplied endogenously or exogenously. Alternatively, HTLV-1 infection may enhance the mutation rate in infected cells.

One may argue that HAM/TSP is a different disease from ATL, and thus the observations made in this report may be specific to HAM/TSP and their families. However, we have reported that 3 of 155 HAM/TSP patients are complicated with ATL, and the incidence of ATL development among HAM/TSP patients is equivalent to that among general HTLV-1 carriers. Therefore, HAM/TSP patients are not an exceptional population in developing ATL. Our previous findings in HTLV-1–infected patients with strongyloidiasis also support that frequent clonal expansion of HTLV-1–infected cells are not unique to HAM/TSP.

The clonally expanded cells observed here were not ATL cells, and the patients did not show any symptoms of ATL, so these cells may not be directly linked to ATL development. However, the highly frequent rate of clonal expansion of HTLV-1–infected cells observed in this study may be an underlying mechanism in triggering smoldering ATL and may give an insight into understanding the early phase of ATL.

REFERENCES


4. Hinuma Y, Nagata K, Misaka M, Nakai M, Matsumoto T,


Frequent clonal proliferation of human T-cell leukemia virus type 1 (HTLV-1)-infected T cells in HTLV-1-associated myelopathy (HAM-TSP)

Y Furukawa, J Fujisawa, M Osame, M Toita, S Sonoda, R Kubota, S Ijichi and M Yoshida