Immunologic Correlates of Spontaneous Lymphocyte Proliferation in Human T-Lymphotropic Virus Infection

By Harry E. Prince, Helen Lee, Eric R. Jensen, Priscilla Swanson, Dawn Weber, Lynda Fitzpatrick, Mary Doyle, and Steven Kleinman

Previously we showed that mononuclear cells from about half of human T-lymphotropic virus (HTLV)-seropositive persons exhibit spontaneous proliferation in vitro. We sought to determine if proliferation was associated with other immunologic changes characteristic of HTLV infection. The parameters assessed were (1) percentages of lymphocytes expressing CD4 and/or CD25 (interleukin-2 receptor), (2) serum levels of soluble CD25, (3) serostatus for other viruses, (4) anti-HTLV antibody levels, and (5) HTLV type determined by polymerase chain reaction or serologic reactivity with type-specific peptides. The proliferation HTLV (PROL') group, proliferation HTLV (PROL') group, and control group showed similar percentages of CD4', CD25', and CD4'CD25' lymphocytes; serum levels of soluble CD25 were also similar. Antibodies to cytomegalovirus, hepatitis B core, and hepatitis C were present in similar proportions of PROL' and PROL groups. However, a significant association was found between spontaneous proliferation and anti-HTLV antibody levels; sera from 67% of PROL' persons, but only 18% of PROL persons, required dilution to yield absorbance values within the linear range of the anti-HTLV antibody assay. In the PROL' group, persons whose sera required the most dilution had proliferative responses significantly higher than those whose sera required no dilution. The PROL' and PROL groups were similar with regard to the relative distribution of HTLV-I and HTLV-II infection. These findings indicate that HTLV-related spontaneous lymphocyte proliferation is related to levels of circulating anti-HTLV antibodies, and characterizes both HTLV-I and HTLV-II infection.

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MATERIALS AND METHODS

Study subjects. HTLV-seropositive persons (N = 134) were identified via screening of donated blood. Results were confirmed by Western blot and radioimmunoprecipitation assays as described. Blood specimens for the studies presented here were collected during a subsequent interview, with the approval of the Institutional Review Board of American Red Cross Blood Services, Los Angeles/Orange Counties Region. None of the study participants showed symptoms of ATL or HAM/TSP. Controls were HTLV-seronegative blood donors.

Spontaneous lymphocyte proliferation assay. Mononuclear cells were obtained by density gradient centrifugation of heparinized venous blood from the 134 HTLV-seropositive persons and 20 controls. These cells were resuspended at 500,000 lymphocytes/mL.

From the American Red Cross Blood Services, Los Angeles, CA; and Abbott Laboratories, North Chicago, IL.

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Address reprint requests to Harry E. Prince, PhD, American Red Cross Blood Services, 1130 S Vermont Ave, Los Angeles, CA 90006.

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in RPMI culture medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine, and 10% pooled human serum. Cells were cultured in flat-bottom microtiter wells (0.2 mL cell suspension per well) for 7 days at 37°C in 5% CO₂. Each well then received 0.5 µCi [³H]-thymidine (Research Products International, Walkersville, MD) and incubation was continued for an additional 4 hours. Cells were then harvested onto glass fiber strips and counted for radioactivity.

To determine if spontaneous proliferation merely reflected better in vitro survival of lymphocytes from HTLV-seropositive persons compared with controls, cells cultured for 7 days were retrieved from microtiter wells, washed, and adjusted to 500,000 viable lymphocytes/mL in fresh culture medium. This suspension was placed in microtiter wells (100,000 cells/well) and [³H]-thymidine incorporation assessed as described.

**CD4 and CD25 cell levels.** The percentages of lymphocytes expressing CD4 and/or CD25 were measured for 40 HTLV-seropositive persons and 20 controls. Whole blood (50 µL) collected in EDTA tubes was placed in each of four polystyrene 12 x 75 mm tubes containing 0.1 mL of phosphate-buffered saline with 1% newborn calf serum (PBS-NCS) and monoclonal antibodies (Becton Dickinson Immunocytometry Systems, San Jose, CA) according to the following scheme: tube 1 = phycoerythrin (PE)-tagged nonspecific mouse IgGl, fluorescein (F)-tagged nonspecific mouse IgGl; tube 2 = PE-anti-CD3; tube 3 = F-anti-CD3; tube 4 = PE-anti-CD25, F-anti-CD4. Following incubation at 4°C for 30 minutes, each tube received 2 mL of lysing agent (0.15 mol/L ammonium chloride) and was incubated for 10 minutes at room temperature. The cells were then washed in PBS-NCS and resuspended in 0.5 mL of the same buffer.

Cell fluorescence was analyzed using a FACScan brand flow cytometer (Becton Dickinson Immunocytometry Systems). Following the establishment of a lymphocyte gate, fluorescence gains were optimized using the cells in tube 1. Fluorescence compensation was then adjusted using the combined contents of tubes 2 and 3. Data for 10,000 ungated events were then collected from tube 1, the tube 2 + 3 combination, and tube 4; lymphocyte fluorescence was then analyzed using FACScan research software. Quadrants were set so that background fluorescence, as assessed using the cells in tube 1, was less than 2%.

**Serum soluble CD25 assay.** Serum levels of soluble CD25 were measured for 52 HTLV-seropositive and 34 control samples using a double-epitope enzyme immunoassay (AMAC, Inc, Westbrook, ME). Absorbance values were converted to picomole values by interpolation from a standard curve.

**Detection of antibodies to other viruses.** Antibodies recognizing cytomegalovirus (CMV) were assessed in serum from the 134 HTLV-seropositive donors using a qualitative latex agglutination assay (Becton Dickinson Microbiology Systems, Cockeysville, MD). Antibodies recognizing hepatitis B core (HBc) proteins were assessed in sera from 129 of the HTLV-seropositive donors using a competitive enzyme immunoassay (Abbott Laboratories, North Chicago, IL). Sera from 109 of the HTLV-seropositive donors were tested for antibodies recognizing hepatitis C virus (HCV) using an enzyme immunoassay (Ortho Diagnostics, Raritan, NJ).

**Screening immunoassay for anti-HTLV antibodies.** Serum specimens from the HTLV-seropositive group (N = 134) were retested for anti-HTLV antibodies using a licensed enzyme immunoassay (Abbott Laboratories). The cutoff absorbance value was calculated following the manufacturer's instructions. The signal/cutoff ratio (SCR) was the quotient obtained by dividing the absorbance value for a given serum sample by the cutoff value. Specimens with absorbance values greater than 2.0 were diluted 1:3, 1:9, 1:27, and 1:81 in HTLV-negative human serum and tested again.

**Determination of HTLV type.** Serum samples from all 134 HTLV-seropositive study subjects and six seronegative controls were tested for antibodies to HTLV-I or -II using a series of synthetic peptide-coated polystyrene beads in a solid-phase enzyme immunoassay. Each peptide represented an area of 20 to 30 amino acids from HTLV envelope or core regions. If the HTLV type of HTLV-seropositive samples could not be determined in the synthetic peptide assay, frozen mononuclear cells, if available, were thawed and further characterized by polymerase chain reaction (PCR) as previously described.

**Statistics.** Differences among proportions were analyzed for significance using the χ² test. Group means were compared using the Mann Whitney U-test. Significance was defined as P < .05.

**RESULTS**

**Spontaneous lymphocyte proliferation.** Shown in Fig 1 are proliferative responses of mononuclear cells for 20 HTLV-seronegative controls and 40 consecutively tested HTLV-seropositive former blood donors. Based on the distribution of proliferative responses in the control group, proliferative responses in the HTLV-seropositive group were scored as proliferation-positive (PROL⁺) if greater than 2,000 cpm, and proliferation-negative (PROL⁻) if ≤2,000 cpm. Thus, in this group of 40 HTLV-seropositive samples, equal proportions (50%) were PROL⁺ and PROL⁻. Cells from a total of 134 HTLV-seropositive individuals were tested for spontaneous lymphoproliferation; 63 (47%) were PROL⁺ and 71 (53%) were PROL⁻.

PROL⁺ cultures did not merely reflect better in vitro survival of lymphocytes from HTLV-seropositive individuals, compared with control individuals (Table 1). DNA synthesis values for day 7 control cell cultures adjusted to 100,000 viable cells per well (viable cell assay results, Table 1) were almost identical to DNA synthesis values of unadjusted cultures (routine assay results, Table 1). Similarly, the proliferative status (PROL⁺ or PROL⁻) of cultures of cells from HTLV-seropositive persons was the same in adjusted and unadjusted cultures.
Lymphocyte CD4 and CD25 expression. The percentages of lymphocytes expressing CD4 and/or CD25 were measured by flow cytometry in a subgroup of 40 HTLV-seropositive persons (20 PROL⁺, 20 PROL⁻) and 20 controls. In all three study groups, the mean percentage of lymphocytes with the indicated phenotype were as follows: CD4⁻CD25⁻ = 8%; CD4⁺CD25⁻ = 21 or 22%; CD4⁻CD25⁺ = 24 or 25. No significant differences were found when comparing the PROL⁺ and PROL⁻ groups, or when comparing each of these groups with the control group.

Absolute lymphocyte counts were available for all but one of the PROL⁺ and one of the PROL⁻ individuals; the mean values were not significantly different (2,449 ± 872 lymphocytes/μL for PROL⁺, 2,452 ± 1,050 for PROL⁻). Further, the proportions of each group with atypical lymphocytes detectable by microscopic examination of differential slides were not significantly different (13 of 62 (21%) of PROL⁺, 14 of 70 (20%) of PROL⁻).

Soluble CD25 levels in serum. Serum levels of soluble CD25 were measured in 52 of the HTLV-seropositive persons (26 PROL⁺, 26 PROL⁻) and 34 controls. The mean values (pmol/L) ± standard deviation were 59 ± 34 for the PROL⁺ group, 72 ± 64 for the PROL⁻ group, and 65 ± 25 for the control group. No significant differences were observed when comparing any combination of two groups.

CMV, HBc, and HCV serostatus. The proportions of PROL⁻ and PROL⁺ groups with antibodies to CMV, HBc, or HCV were not significantly different. These observed proportions were as follows: CMV⁺ = 72% of PROL⁺, 81% of PROL⁻; HBc⁺ = 21% of PROL⁺, 21% of PROL⁻; HCV⁺ = 22% of PROL⁺, 16% of PROL⁻.

Anti-HTLV screening assay. All 134 serum specimens from HTLV-seropositive persons were retested for anti-HTLV antibodies using a licensed enzyme immunoassay (Abbott). As shown in Table 2, a significantly higher proportion of serum samples from the PROL⁻ group exhibited absorbance values less than 2.0 with SCR less than 3.0, compared with samples from the PROL⁺ group. This difference was also reflected in the finding that the proportion of specimens with absorbance values above the upper limit of the assay (ie, >2.0) was significantly higher in the PROL⁺ group than in the PROL⁻ group (line 4, Table 2). When serial threefold dilutions of these serum samples with absorbance values greater than 2 were retested, a significantly higher proportion of those from the PROL⁻ group, compared with the PROL⁺ group, exhibited absorbance values on scale (<2) at a 1:3 dilution (line 5, Table 2). Thus, circulating anti-HTLV antibody levels were generally higher in the PROL⁺ group than the PROL⁻ group.

Within the PROL⁺ group, there was a trend toward higher spontaneous proliferative responses in association with higher anti-HTLV antibody levels. As shown in Fig 2, proliferative responses were significantly higher in the subgroup whose sera had to be diluted 1:81 to obtain on-scale absorbance values, compared with the subgroup whose sera required no dilution.

Determination of HTLV type. Serum from the 134 HTLV-seropositive study subjects (63 PROL⁺ and 71 PROL⁻) were tested for reactivity to synthetic peptides specific for HTLV-I or HTLV-II. By this method, 121 of the 134 specimens (90%) were identified as HTLV-I or HTLV-II (Table 3). As an internal positive control for the peptide assay, 19 of the 134 sera were from individuals previously typed by PCR as HTLV-II, and 17 of the 19 were identified as HTLV-II by the peptide assay; two were negative by the peptide assay. Similarly, six serum samples from HTLV-I⁺ persons, as determined previously by PCR, were all typed as HTLV-I by the peptide assay. All of six seronegative control sera gave negative results in the peptide assay.

Frozen cells were available from 11 of the remaining 13 untyped HTLV-seropositive specimens, and these were subjected to PCR analysis to determine the HTLV type;

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**Table 1. Spontaneous Proliferation Does Not Merely Reflect Improved In Vitro Survival of Cells From HTLV-Infected Individuals**

<table>
<thead>
<tr>
<th>Group</th>
<th>Specimen</th>
<th>Routine Assay</th>
<th>Viable Cell Assay</th>
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<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>878</td>
<td>706</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>149</td>
<td>312</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>183</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1,262</td>
<td>625</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>466</td>
<td>573</td>
</tr>
<tr>
<td>HTLV⁺</td>
<td>1</td>
<td>393</td>
<td>363</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,548</td>
<td>394</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13,052</td>
<td>5,216</td>
</tr>
<tr>
<td></td>
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<td>78,204</td>
<td>180,062</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>11,659</td>
<td>17,396</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>68,810</td>
<td>74,674</td>
</tr>
</tbody>
</table>

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**Table 2. Anti-HTLV Antibody Screening Test Results**

<table>
<thead>
<tr>
<th>Abbott Test Result</th>
<th>PROL⁻ (N = 71)</th>
<th>PROL⁺ (N = 63)</th>
<th>χ² P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs &lt; 2, SCR 1.0-2.0</td>
<td>30 (42.3)</td>
<td>10 (15.9)</td>
<td>.0019</td>
</tr>
<tr>
<td>Abs &lt; 2, SCR 2.0-3.0</td>
<td>15 (21.1)</td>
<td>4 (6.3)</td>
<td>.0144</td>
</tr>
<tr>
<td>Abs &lt; 2, SCR &gt; 3.0</td>
<td>13 (18.3)</td>
<td>7 (11.1)</td>
<td>.2431</td>
</tr>
<tr>
<td>Abs &gt; 2</td>
<td>13 (18.3)</td>
<td>42 (66.7)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>&lt; 2 at 1:3</td>
<td>5 (38.5)*</td>
<td>4 (6.3)*</td>
<td>.0137</td>
</tr>
<tr>
<td>&lt; 2 at 1:9</td>
<td>4 (30.8)*</td>
<td>14 (33.3)*</td>
<td>.9633</td>
</tr>
<tr>
<td>&lt; 2 at 1:27</td>
<td>3 (23.1)*</td>
<td>11 (26.2)*</td>
<td>.8218</td>
</tr>
<tr>
<td>&lt; 2 at 1:81</td>
<td>1 (7.7)*</td>
<td>13 (31.0)*</td>
<td>.0925</td>
</tr>
</tbody>
</table>

Abbreviation: Abs, absorbance value.

*Percent of the group with Abs greater than 2.
As summarized in Table 3, the proportion of HTLV-I+ individuals who exhibited proliferation (13 of 26 [50.0%]) was similar to the proportion of HTLV-II+ individuals exhibiting proliferation (48 of 103 [46.6%], P = .7565). Likewise, the distributions of HTLV-I versus HTLV-II infection within the PROL+ and PROL- groups were similar. Thus, spontaneous lymphocyte proliferation occurred in association with both HTLV-I and HTLV-II infection. Further, there was no stratification of proliferative responses in relation to HTLV type; the mean proliferative response for the 13 HTLV-I+ PROL+ persons (28,349 cpm ± 18,866) was not significantly different from the mean value for the 48 HTLV-II PROL+ persons (23,998 cpm ± 29,740).

Table 3. Spontaneous Proliferation in Relation to HTLV Type

<table>
<thead>
<tr>
<th>Type</th>
<th>Method</th>
<th>PROL</th>
<th>PROL+</th>
<th>Fraction of HTLV Type PROL+</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Peptide</td>
<td>12</td>
<td>13</td>
<td>50.0%</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Peptide</td>
<td>51</td>
<td>45</td>
<td>46.6%</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>55</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

Fraction of PROL group HTLV+ 19.1% 21.3%

N = 129, and does not include data for five HTLV-seropositive individuals whose HTLV type could not be determined.

DISCUSSION

Our findings show that mononuclear cells from roughly half of a large group of asymptomatic HTLV-seropositive donors from the Los Angeles area exhibit spontaneous lymphocyte proliferation when cultured in vitro for 7 days, confirming our previous findings for a smaller group of HTLV-seropositive individuals. Two other groups have reported spontaneous lymphoproliferation in asymptomatic HTLV-seropositive persons from Japan and the Caribbean (HTLV-I endemic areas). However, in these studies only mean values for proliferative responses were presented; no mention was made of the proportion of individuals in the carrier groups exhibiting responses within the normal range.

The mechanism responsible for HTLV-related spontaneous lymphocyte proliferation remains unclear. Studies by Gazzolo et al and Duc Dodon et al have shown that HTLV-I envelope glycoproteins are mitogenic for normal human T cells. The time course of this response and phenotype of responding cells are similar to the spontaneous lymphoproliferative responses described here. We and others thus postulate that HTLV-related spontaneous lymphocyte proliferation reflects a response of lymphocytes to an intrinsic mitogenic or transactivating factor produced in association with HTLV infection (HTLV envelope peptides or Tax?). Studies are currently underway to identify the cellular source of this putative factor and to characterize the cell-cell interactions regulating the response.

We sought to determine if the presence or absence of a spontaneous proliferative response was related to other immunologic changes characterizing overt HTLV infection. The assay system used to measure CD4/CD25 expression (PE-anti-CD25 staining measured with a FACScan brand flow cytometer) has been shown to be much more sensitive than other assay systems, thus yielding %CD25+ lymphocyte values greater than previously thought. In support of our earlier findings for HTLV-seropositive donors not stratified by spontaneous proliferation results, levels of lymphocytes expressing CD4 and/or CD25 were normal in both the PROL+ and PROL- HTLV groups. Likewise, serum levels of soluble CD25 were normal in these two groups; this finding is consistent with those of Yasuda et al, who found normal soluble CD25 levels in asymptomatic HTLV-I carriers.

Because the prevalence of spontaneous lymphocyte proliferation in HTLV-infected carriers (about 50%) was similar to the prevalence of anti-CMV antibodies in the general blood donor population, we asked if coincident CMV infection was associated with spontaneous proliferation. The data demonstrated that anti-CMV antibodies were present in most of the HTLV-seropositive persons, regardless of proliferation status. Similarly, no significant associations were observed between proliferation status and the prevalence of antibodies recognizing HBC or HCV. Thus, coincident infection by these viruses did not appear...
to play a significant role in HTLV-associated spontaneous lymphocyte proliferation.

When assessing the relationship between spontaneous proliferation and circulating anti-HTLV antibodies, we documented a highly significant association. Whereas most persons in the PRL- group had a circulating anti-HTLV antibody concentration higher than that detected in the range of linearity in the Abbott screening assay, most persons in the PRL- group did not. Further, PRL- persons with very high anti-HTLV antibody levels exhibited higher spontaneous lymphoproliferative responses than PRL+ persons with lower antibody levels. Thus, higher levels of circulating anti-HTLV antibodies were associated with HTLV-related spontaneous lymphoproliferation. This association observed for asymptomatic HTLV carriers is consistent with data published by Itoyama et al for HAM/TSP patients; all 14 HAM/TSP patients had very high levels of HTLV-I antibodies, and lymphocytes from all 14 patients showed spontaneous proliferation.

The conditions responsible for the association between levels of circulating anti-HTLV antibodies and spontaneous proliferation remain unknown. High levels of circulating anti-HTLV antibodies are generally associated with a higher frequency of HTLV-infected cells, as judged by levels of signal generated in PCR analysis (Dr Helen Lee, unpublished observations). Thus, high levels of anti-HTLV antibodies may reflect a greater viral load. Our data showing a significant association between anti-HTLV antibody levels and spontaneous proliferation thus suggest that the presence or absence of spontaneous proliferation also reflects differences in viral load. In support of this hypothesis, about 10% of mononuclear cells from HAM/TSP patients contain HTLV-I proviral DNA, and lymphocytes from essentially all HAM/TSP patients appear to exhibit spontaneous proliferation. In contrast, only 0.1% to 2% of lymphocytes from asymptomatic HTLV-I carriers contain proviral DNA, and we show here that lymphocytes from only half of asymptomatic carriers show spontaneous proliferation. A higher frequency of HTLV-infected cells would be expected to lead to greater levels of the putative mitogenic factor responsible for cell activation.

An important finding arising from the results of the type determination assay is that a proliferative response was observed in similar proportions of HTLV-I+ and HTLV-II+ individuals. Thus, spontaneous proliferation was clearly not limited to a specific HTLV type causing infection. A recent abstract by Blattner et al presented similar findings. If future studies identify a link between spontaneous proliferation and clinical progression of HTLV-associated disease, then disease development should be expected in HTLV-II, as well as HTLV-I, infection.

REFERENCES


Immunologic correlates of spontaneous lymphocyte proliferation in human T-lymphotropic virus infection

HE Prince, H Lee, ER Jensen, P Swanson, D Weber, L Fitzpatrick, M Doyle and S Kleinman