Interleukin 4 stimulates infection and temporary growth of human neonatal lymphocytes exposed in vitro to human T-lymphotropic virus type I, but fails to substitute for interleukin 2 in the immortalization of infected cultures

Antonio Mastino,1 Sandro Grelli,2 Cartesio Favalli,3, 4 Claudia Matteucci,3 Marco De Carli,5 Enrico Garaci3 and Beatrice Macchi3, 4

1 Institute of Microbiology, Faculty of Science, University of Messina, Messina, Italy
2 Institute of Experimental Medicine, CNR, Rome, Italy
3 Department of Experimental Medicine and Biochemical Science, University of Rome ‘Tor Vergata’, Via di Tor Vergata 135, 00133 Rome, Italy
4 IRCCS, S. Lucia, Rome, Italy
5 Institute of Internal Medicine and Immunoallergology, University of Florence, Florence, Italy

It has been shown that interleukin 4 (IL-4) stimulates the proliferation of cells from patients affected by adult T-cell leukaemia, the haematological malignancy aetiologically associated with human T-lymphotropic virus type I (HTLV-I). In the present study, human neonatal lymphocytes were exposed to HTLV-I in vitro in the presence of IL-4. The results showed that: (i) cultures exposed to HTLV-I in the presence of either IL-4 or IL-2 bound IL-4; (ii) IL-4 did not substitute for IL-2 as a growth factor in cell lines previously infected and maintained in IL-2; (iii) cultures exposed to HTLV-I and maintained in IL-4 or IL-2 became infected; and (iv) IL-4 sustained the growth of HTLV-I-infected cultures for a maximum of 14 weeks. Moreover, HTLV-I-infected cultures grown in IL-4 showed upregulation of the IL-4 message and lower expression of HLA-DR and CD25 when compared with counterpart cultures maintained in IL-2. These results suggest that continuous growth of T-lymphocytes induced in vitro by HTLV-I infection, at least temporarily, requires signals specifically provided by IL-2 and not by IL-4.

Introduction

Human T-lymphotropic virus type I (HTLV-I) is aetiologically linked to adult T-cell leukaemia/lymphoma (ATLL; Poiesz et al., 1981; Yoshida et al., 1982; Seiki et al., 1982) and has also been associated more recently with many other disorders. These include tropical spastic paraparesis, a slow progressive myelopathy with signs of spinal cord atrophy and demyelination prevalent both in the Tropics (Ceroni et al., 1988) and in Japan, where it is known as HTLV-I-associated myelopathy (Osame et al., 1986), as well as uveitis and arthropathies (Nishioka et al., 1989; Sagawa et al., 1995). Although the virus infects a variety of target cells of lymphoid and non-lymphoid origin in vitro (Macchi et al., 1987; Hoxie et al., 1985; Graziani et al., 1993), it seems to immortalize (factor-independent growth) and occasionally transform (factor-independent growth) mainly T-lymphocyte cultures (Kimata & Ratner, 1991). T-lymphocytes infected in vitro by HTLV-I and grown in the presence of interleukin 2 (IL-2) express the α-chain of the high-affinity receptor for IL-2, like T-lymphocytes from ATLL patients, and are IL-2-dependent for a prolonged period in culture. Since the HTLV-I protein Tax is known to upregulate expression of both the IL-2 and the IL-2 receptor (IL-2R) α-chain (Siekevitz et al., 1987), an autocrine/paracrine IL-2/IL-2R loop might be considered as a major mechanism involved in immortalization/transformation of T-lymphocytes (Franchini, 1995). However, expression of the IL-2 gene was undetectable in HTLV-I-transformed cultures (Arya et al., 1984). Since HTLV-I-infected cells have been shown to express various cytokines and growth factors constitutively, it may be that interleukins other than IL-2 could be involved in the immortalization/transformation process driven by HTLV-I.

IL-4 is a pleiotropic, immunoregulatory lymphokine, pre-
dominantly produced by T-cells, mast cells and basophil cells. In vitro studies have demonstrated that IL-4 has a broad range of functions with respect to B-cells, macrophages and mast cells (Paul, 1991). In addition, IL-4 has an IL-2-independent growth-promoting effect on activated T-cells (Spits et al., 1987; Brown et al., 1988). Furthermore, it has been reported that IL-4 could play a central, although probably not sufficient, role in the acquisition of a Th2 phenotype, which is mainly characterized by production of IL-4, IL-5, IL-6 and IL-10 in murine and human lymphocytes (Swain, 1991; Maggi et al., 1992). It has been shown that cells from some ATLL patients proliferate in response to IL-4 (Uchiyama et al., 1988), suggesting that activation of the IL-4 pathway in leukaemic cells from some patients could occur following HTLV-I infection. Furthermore, ATLL cells were found to exhibit significant expression of the IL-4 receptor (IL-4R) on their surfaces without additional stimulation (Mori et al., 1996). Finally, it has been reported that IL-4 plays a regulatory role in the production of IL-6 and IL-1 in ATLL cells (Mori et al., 1993, 1995). However, it is not clear whether IL-4 has an effect on the establishment and progression of HTLV-I infection in naive human lymphocytes and in the process of HTLV-I-driven immortalization/transformation of T-lymphocytes.

In the present study, we have explored whether IL-4 stimulates HTLV-I infection of human neonatal T-cells in vitro and the successive expansion and growth of infected cells. We have also investigated the influence of IL-4 on phenotype and on the pattern of cytokine production in HTLV-I-infected lymphocytes in vitro.

**Methods**

**In vitro infection with HTLV-I.** Mononuclear cells collected from cord blood (CBMC) drawn at terminal delivery, were separated by Ficoll–Hypaque density gradients (Pharmacia). After being washed in PBS, a total of either $5 \times 10^6$ or $1 \times 10^7$ CBMC at an initial concentration of $1 \times 10^6$ cells per ml was co-cultured with lethally irradiated MT-2 cells at an acceptor:donor ratio of 3:1. Irradiation was performed by exposing the cells to 120 Gy in a Caesium Gamma Cell 1000 (Atomic Energy of Canada). These procedures, adopted as standard method for HTLV-I cell-to-cell transmission in our laboratory as previously reported (Macchi et al., 1993), have been experimentally selected as the most suitable conditions for HTLV-I transmission while avoiding MT-2 overgrowth, and derive from techniques previously described by other authors (Yamamoto et al., 1982; Popovic et al., 1983). MT-2 is a chronically HTLV-I-infected cell line derived from cord blood infected with the virus from an ATLL patient (Miyoshi et al., 1984). Co-cultures were grown in RPMI 1640 medium supplemented with 20% FCS, glutamine, penicillin–streptomycin (all from Gibco). Human recombinant IL-2 (Cetus) or human recombinant IL-4 (British Bio–Technology) was added weekly to the culture medium at a final concentration of 20 U/ml. Cultures were passaged weekly; cell growth was monitored by evaluating living cells using a trypan blue dye exclusion test and cell concentration was re-adjusted to $1 \times 10^5$ cells per ml. Results of cell growth were expressed by calculating the theoretical total cell number (TTCN) for the first week and TTCN at previous weekly passage × millions of cells per ml before readjustment for the following weeks. Aliquots of irradiated MT-2 cells, utilized as donor cells for infection and kept separately in culture medium for the duration of the experimental culture, showed no evidence of growth. HTLV-I-infected cell lines grown in the presence of IL-2 or IL-4 were designated CBXXMT/IL-2 or CBXXMT/IL-4, respectively, where XX corresponds to a progressive cell number which identifies the donor.

**IL-2 and IL-4 binding assay by flow cytometry analysis.** In order to determine the percentage of cells bearing receptors for IL-2 or IL-4 and the receptor density for these cytokines on cell surfaces, biotinylated IL-2 and IL-4 were used in combination with avidin–FITC (Fluorokine kits, R&D Systems) in flow cytometric analysis. In order to remove residual growth factors, cells were resuspended in fresh RPMI 1640 plus 5% FCS and pre-incubated for 1 h at 37 °C. Cells were then washed twice in PBS and resuspended at a final concentration of $2 \times 10^5$ in 25 µl of the same PBS. Biotinylated IL-2 or IL-4 (10 µl) was added to the cell suspension followed by incubation for 60 min at 4 °C. Cells were then washed twice, 10 µl avidin–FITC was added and cells were further incubated at 4 °C for 30 min. Cells were again washed twice, resuspended in PBS and analysed by flow cytometry for single fluorescence. Control cells were treated with avidin–FITC alone. Gates were set according to standard light scattering criteria for discriminating living cells from debris, dead cells and cell aggregates. For each sample, 5000 events were evaluated.

**mRNA extraction.** mRNA was extracted from $5 \times 10^6$ cells that had been previously washed in PBS. Isolation of poly(A)$^+$ RNA was performed using a QuickPrep MicromRNA purification kit (Pharmacia). Briefly, cells were extracted in a buffered solution containing 4 M guanidinium thiocyanate, ensuring rapid inactivation of endogenous RNases. The extract was then partially purified by centrifugation with an elution buffer. The supernatant was subsequently transferred to a microcentrifuge tube containing oligo(dT)-cellulose (25 mg/ml in a storage buffer containing 0.15% Kathon CG). Poly(A)$^+$ RNA bound to the oligo(dT)–cellulose and was then purified by a series of washes in high and low salt buffers. Finally, the polyadenylated material was eluted with elution buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA) and precipitated by adding 2 M sodium acetate, 95% ethanol and by placing the sample at −20 °C. The collected mRNA was ultimately resuspended in an appropriate volume of elution buffer. After 9 weeks of culture, in order to test MT-2-irradiated cells for virus replication, cell lysates were prepared for reverse transcriptase–PCR.

**RT–PCR.** Total RNA (2 µg) was reverse transcribed into cDNA in 25 µl of reaction mix as follows: 50 ng of mRNA was incubated with a mix containing a final dilution of 1 × reverse transcriptase (RT) buffer, 1 mM dNTPs (Pharmacia), 1.5 µg oligo(dT) (New England Biolabs), 50 U recombinant RNase inhibitor (Boehringer Mannheim), 10 mM DTT (Sigma), 25 U Moloney murine leukaemia virus RT (New England Biolabs) for 1 h at 37 °C. The reaction mix was then incubated at 95 °C for 5 min, in order to inactivate RT and then chilled on ice. cDNA (3 µl) was amplified by PCR in a total volume of 50 µl containing a final dilution of 1× PCR buffer, 0.2 mM dNTPs, 0.5 µM specific primer pair and 2.5 U of Taq polymerase (Boehringer Mannheim). Samples were subjected to 30 cycles of PCR amplification, each cycle consisting of 30 s at 94 °C (DNA denaturation), 30 s at 55 °C (primer annealing) and 15 s at 72 °C (primer extension) in a Perkin Elmer Cetus DNA thermal cycler 2400 (Perkin Elmer). Following the final cycle, samples were incubated at 72 °C for 7 min to ensure completion of the
Table 1. Primers and probes used for PCR analysis

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Position</th>
<th>Probe</th>
<th>Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPX3</td>
<td>(5096–5115)+</td>
<td>RPXPR1*</td>
<td>(5175–5183)</td>
<td>Kinoshita * et al. (1989)</td>
</tr>
<tr>
<td>RPX4</td>
<td>(7357–7338)–</td>
<td></td>
<td>(7302–7312)</td>
<td>Clontech</td>
</tr>
<tr>
<td>IL-2</td>
<td>(74–99) +</td>
<td></td>
<td>(303–326)</td>
<td>Clontech</td>
</tr>
<tr>
<td>IL-4</td>
<td>(378–353)–</td>
<td></td>
<td>(303–326)</td>
<td>Takashi et al. (1986)</td>
</tr>
<tr>
<td>IL-6</td>
<td>(91–110) +</td>
<td></td>
<td>(399–420)</td>
<td>Genis et al. (1992)</td>
</tr>
<tr>
<td>IL-10</td>
<td>(317–337) +</td>
<td></td>
<td>(7357–7338)</td>
<td>Kinoshita et al. (1989)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>(476–455)–</td>
<td></td>
<td>(5096–5115)</td>
<td>Genis et al. (1992)</td>
</tr>
<tr>
<td>β-Globin</td>
<td>(647–616)–</td>
<td></td>
<td>(979–960)</td>
<td>Clontech</td>
</tr>
<tr>
<td></td>
<td>(14–33) +</td>
<td></td>
<td>(472–501)</td>
<td>Seiki et al. (1985)</td>
</tr>
<tr>
<td></td>
<td>(123–104)–</td>
<td></td>
<td>(63–822)</td>
<td></td>
</tr>
</tbody>
</table>

* The probe corresponds to the sequences surrounding the second splice junction site at Tax/Rex mRNA.

The primers RPX3 and RPX4 specifically recognize the Tax/Rex region of HTLV-I. The Tax/Rex region is expressed as doubly spliced mRNA; RPX3 and RPX4 primers are located upstream and downstream, respectively, of the second junction site for Tax/Rex mRNA. mRNA for IL-2 was subjected to 30 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 2 min). mRNAs for IL-4, IL-6, TNF-α, and IFN-γ were amplified in 30 cycles by denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 2 min (see Table 1 for primer sequences). After 30 cycles an additional extension step was performed at 72 °C for 7 min. The samples were also amplified with primers specific for β-globin as an internal control.

Liquid hybridization. Amplified DNA was analysed by liquid hybridization (Ehrlich et al., 1990). Samples were probed using specific 32P-end-labelled oligonucleotides (see Table 1). The 32P-end-labelling of the probes was performed with 10 pM of probe, 30 μCi of γ-32P]ATP (NEN) and 2 U of polynucleotide kinase (New England Biolabs). The reaction mix was incubated for 1 h at 37 °C and unincorporated radioactivity was eliminated by Sephadex G-25 chromatography (Boehringer Mannheim). A reaction mix containing 1.5 M NaCl, 25 mM EDTA and 32P-end-labelled probes was added to the samples which were then denatured for 5 min at 95 °C and annealed at 55 °C for 15 min. A gel-loading dye (15% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to the samples and they were loaded onto a 5% polyacrylamide gel and run in 1× TBE (0.089 M Tris–borate, 0.089 M boric acid, 0.002 M EDTA). The product that was amplified using IFN-γ primers was separated on a 5% gel and the products of all other primers were amplified on an 8% gel. Following electrophoresis, the gel was then dried and exposed to Kodak XAR-5 film for autoradiography.

Analysis of surface molecules. The following mouse anti-human monoclonal antibodies (MAbs) were used for two colour analysis: phycoerythrin (PE)-conjugated CD4 and CD25, and FITC-conjugated CD8 and HLA-DR (Becton–Dickinson). Antibodies were combined as follows: CD4/CD8; and CD25/HLA-DR. Staining was performed at 4 °C for 30 min. After treatment, cells were washed twice in PBS containing 0.02% sodium azide and used immediately. Flow cytometric analysis was performed using a FACScan (Becton–Dickinson). During analysis, markers were set to indicate boundaries limiting 98–99% of the background events in the respective control sample. Background percentage positivity was subtracted from the corresponding experimental samples. Cells to be evaluated were gated on forward- versus side-angle scatter, according to standard criteria, in order to exclude debris, dead cells, giant cells and cell aggregates and to include living lymphoid cells. For each sample, 5000 events were evaluated.

Results

Binding of IL-4 in IL-2-dependent HTLV-I infected cell lines

To assess whether cultures infected with HTLV-I in vitro expressed IL-4R, two CD4+, IL-2-dependent cell lines, obtained from two different normal neonatal donors, were examined. These cell lines were maintained in culture for at least 25 weeks before testing for their ability to bind IL-4. Fig. 1 shows that 70% of cells from the representative cell line CB05MT/IL-2 were able to bind FITC-conjugated IL-4 as determined by flow cytometry (Fig. 1 C). As expected, the cell lines also bound IL-2 (Fig. 1 B) showing a mean fluorescence value slightly higher than that of IL-4. In addition, the corresponding uninfected CB05 cell line grown in IL-2, without any additional stimulation, was checked for IL-4 binding after 3 weeks of culture. The timing of testing of the uninfected cell line was chosen such that it preceded the rapid decrease in cell growth followed...
Fig. 1. Analysis by flow cytometry of IL-2 and IL-4 binding on the surface of cells from a long-term infected cell line maintained in IL-2 and from the uninfected counterparts at 26 and 3 weeks from the onset of the culture, respectively. Cells from the CB05MT/IL-2 or the CB05/IL-2 cell lines were conjugated with biotinylated IL-2 or IL-4 and successively, as a second step, with an avidin–FITC reagent. Histograms of (A) CB05MT/IL-2 labelled with avidin–FITC alone, (B) CB05MT/IL-2 labelled with FITC–IL-2, (C) CB05MT/IL-2 labelled with FITC–IL-4, (D) CB05/IL-2 labelled with avidin–FITC alone, (E) CB05/IL-2 labelled with FITC–IL-2 and (F) CB05/IL-2 labelled with FITC–IL-4.

Fig. 2. Effect of IL-4 addition on the growth of a long-term infected cell line maintained in IL-2. The CB05MT/IL-2 was divided in two subcultures and in one of them IL-2 was replaced by IL-4 at 20 U/ml. Three weeks later cultures in IL-4 stopped growing; however, this effect was reverted by re-addition of IL-2 at 20 U/ml. Solid line: addition of IL-2 to the culture. Dashed line: addition of IL-4.

Growth of IL-2-dependent HTLV-I infected cell lines in IL-4

To assess whether IL-4 could be used as a growth factor by IL-2-dependent cell lines immortalized in vitro by HTLV-I, which was suggested by their ability to bind IL-4, the CB05MT/IL-2 cell line was divided into two subcultures; in one of them IL-2 was replaced by the same amount of IL-4. The subculture in IL-4 was maintained for 3 weeks. Results shown in Fig. 2 indicate that IL-2-dependent cells infected with HTLV-I ceased to grow 3–4 weeks after IL-2 had been replaced by IL-4. However, this effect was partly reversed when IL-4 was replaced by IL-2 in part of the subculture.

Growth of CBMC exposed to HTLV-I in the presence of IL-2 or IL-4

The next step was to verify whether fresh CBMC that had been exposed to HTLV-I were able to respond to IL-4 when they were conditioned by this cytokine at the time of exposure to the virus. To this end, cultures were exposed to HTLV-I by co-cultivation with irradiated MT-2 cells and then divided into two aliquots to which either IL-2 or IL-4 was added. Fig. 3 shows the growth curves of HTLV-I-infected cell lines, obtained from nine different donors, maintained in either IL-4 or IL-2. During the first 3–4 weeks post-infection (p.i.), all the cultures grew at a similar rate whether exposed to IL-4 or IL-2, except in one case where the culture grew better in the...
IL-4 and HTLV-I infection in vitro

Fig. 3. Cell-growth curves of neonatal mononuclear cells isolated from nine different donors, infected with HTLV-1 in vitro and maintained in IL-2 (□), in IL-4 (■) or in absence of factors (●). Viable cells from infected cultures were counted at the indicated times p.i. by trypan blue exclusion test. Results are expressed as theoretical total cell number calculated for each culture on the basis of weekly adjustment of cell concentrations. (A) CB23MT; (B) CB25MT; (C) CB56MT; (D) CB71MT; (E) CB72MT; (F) CB74MT; (G) CB75MT; (H) CB73MT; (I) CB30MT.

Fig. 4. Analysis by flow cytometry of IL-2 and IL-4 binding on the surface of cells grown in IL-4. Cells from CB25MT were conjugated as previously described. Histograms of (A) CB25MT/IL-4 labelled with avidin–FITC alone, (B) CB25MT/IL-4 labelled with FITC–IL-2 and (C) CB25MT/IL-4 labelled with FITC–IL-4. This experiment is representative of four experiments performed.

presence of IL-4 (Fig. 3A). Results obtained in the corresponding uninfected CBMC showed that IL-4 acted as a very efficient CBMC growth factor during the same phase of culture (data not shown). Indeed, in some cases, uninfected cultures kept in IL-4 with no additional stimulus grew longer than the corresponding uninfected cultures maintained in IL-2, i.e. for up to 7 weeks. From 3–4 weeks onwards, the number of surviving cells in HTLV-I-infected cell lines maintained in IL-4 slowly decreased and all cultures eventually died. In fact, in contrast to cell lines maintained in IL-2, they survived in
Table 2. Phenotype of uninfected and HTLV-1 infected neonatal lymphocytes grown in IL-2 or IL-4

Uninfected human neonatal lymphocytes, grown in complete medium containing either IL-2 (20 U/ml) or IL-4 (20 U/ml), were harvested after 4 weeks cultivation and analysed by flow cytometry for surface molecule expression. HTLV-1-infected human neonatal lymphocytes, cultured in the same conditions as uninfected cultures, were harvested at 8 weeks p.i. and analysed by flow cytometry for surface molecule expression. The following anti-human MAbs were used: PE-conjugated CD4, CD25, and FITC-conjugated CD8, HLA-DR. Staining was performed at 4 °C for 30 min.

<table>
<thead>
<tr>
<th>Cultures*</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4/CD8</th>
<th>CD25</th>
<th>HLA-DR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+IL-2</td>
<td>+IL-4</td>
<td>+IL-2</td>
<td>+IL-4</td>
<td>+IL-2</td>
</tr>
<tr>
<td>CB56</td>
<td>50</td>
<td>13</td>
<td>59</td>
<td>44</td>
<td>4</td>
</tr>
<tr>
<td>CB56MT</td>
<td>94</td>
<td>96</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>CB70</td>
<td>28</td>
<td>72</td>
<td>29</td>
<td>29</td>
<td>1</td>
</tr>
<tr>
<td>CB70MT</td>
<td>91</td>
<td>82</td>
<td>9</td>
<td>35</td>
<td>3</td>
</tr>
<tr>
<td>CB72</td>
<td>49</td>
<td>75</td>
<td>9</td>
<td>30</td>
<td>9</td>
</tr>
<tr>
<td>CB72MT</td>
<td>94</td>
<td>66</td>
<td>4</td>
<td>42</td>
<td>2</td>
</tr>
<tr>
<td>CB74</td>
<td>45</td>
<td>56</td>
<td>20</td>
<td>51</td>
<td>2</td>
</tr>
<tr>
<td>CB74MT</td>
<td>74</td>
<td>73</td>
<td>28</td>
<td>42</td>
<td>3</td>
</tr>
</tbody>
</table>

* Cultures set up from four different donors are designated by progressive number. HTLV-1-infected cultures are designated by the progressive number plus MT.
culture for a maximum of 14 weeks (Fig. 3E, F). On the other hand, when neonatal mononuclear cells infected with HTLV-I in vitro were cultured in complete medium alone as a control, without the addition of either IL-2 or IL-4, viable cell numbers rapidly decreased until all cultures eventually died without showing any proliferating response to virus exposure (Fig. 3A–H). As with IL-2-dependent cell lines infected by HTLV-I, infected cells maintained in IL-4 showed highly efficient binding of IL-2, as observed in the representative cell line CB25MT/IL-4, 5 weeks p.i. (Fig. 4). On the other hand, as shown in the same experiment, only a fraction of HTLV-I-infected neonatal cells maintained in IL-4 retained the initial capacity of binding IL-4 (80% positive cells, 1 week p.i.) with the same efficiency as IL-2. All infected cultures maintained in IL-2, with the exception of that reported in Fig. 3A, continued to grow and were maintained for at least 3 months, longer than corresponding HTLV-I-infected cultures grown in IL-4. In a few experiments, when IL-4-conditioned cultures infected with HTLV-I ceased to grow, IL-4 was replaced by IL-2. In this case, cultures resumed a high proliferation rate. However, the recovery in cell growth was only transient and after 1 or 2 weeks all cultures showed an irreversible decline and died (data not shown).

**Virus detection in HTLV-I-infected cell lines grown in IL-2 or IL-4**

In order to ascertain whether the different growth of cultures exposed to HTLV-I and kept in IL-2 or IL-4 could be attributable to the influence of IL-4 on the establishment and progression of HTLV-I infection in CBMC, expression of the virus was monitored at intervals by RT–PCR. Results of a representative experiment, performed 9 weeks p.i., are illustrated in Fig. 5. Viral mRNA was present in HTLV-I-infected neonatal lymphocytes cultured in either IL-2 or IL-4 (Fig. 5a). The housekeeping β-globin gene was expressed equally in both cultures (Fig. 5a). Irradiated MT-2 cells alone, maintained as long as HTLV-I-exposed cultures, gave no amplified products when processed by RT–PCR 9 weeks after the irradiation (Fig. 5b). Similarly, in either IL-2- or IL-4-conditioned cell lines infected with HTLV-I comparable levels of viral mRNA were detected at all times tested after infection (data not shown).

**Phenotype of HTLV-I-infected neonatal lymphocytes grown in IL-2 or in IL-4**

Phenotypic analysis of cultures kept in different conditions is reported in Table 2. A higher level of expression of CD8 was observed in three out of four infected cell lines maintained in IL-4 compared with those kept in IL-2. This was mainly related to the fact that a double positive CD4+/CD8+ population progressively emerged. The emergence of CD4+/CD8+ cells was also evident, albeit at a lower level, in uninfected control cultures maintained in IL-4. In two out of four cultures (CB70MT and CB74MT) CD25 was expressed at a lower level in cultures maintained in IL-4 compared with their counterparts maintained in IL-2. The HLA-DR marker was consistently lower in all of the four infected cell lines tested which had been maintained in IL-4, compared with those maintained in IL-2.

**Expression of cytokine mRNA**

Infected cell lines were also assayed for expression of various cytokine mRNAs by RT–PCR analysis. A constant cell number of $5 \times 10^6$ was used for RNA extraction and a constant amount of mRNA from each extract was utilized for reverse transcription to cDNA, as demonstrated by β-globin expression in Fig. 5a. As shown in Fig. 6, in a representative cell line at 9 weeks p.i., HTLV-I-infected neonatal lymphocytes...
supported in their growth by IL-2 expressed mRNA for IL-6, IL-2, IFN-γ, and to a lesser extent, TNF-α, whereas the message for IL-4 was virtually absent. The pattern of cytokine expression in corresponding infected cultures maintained in IL-4 was very different. In fact, IL-6, IL-2, IFN-γ and TNF-α mRNAs were expressed either very poorly or not at all, whereas the message for IL-4 was present at a high level. In HTLV-I-infected cell lines maintained in either IL-2, IL-4 or IL-10, expression was undetectable.

Discussion

The ultimate mechanisms underlying the molecular basis of the leukaemogenic action triggered by HTLV-I infection have not yet been elucidated. Paradoxically, one of the most intriguing questions remains why some 96–98% of subjects infected by HTLV-I in endemic areas of Japan do not develop ATLL (Tokudome et al., 1989). This could be explained by many different factors, but one of the most convincing may be that infection must be considered as only the first of many steps in the development of leukaemogenesis (Hollsberg & Hafler, 1993).

The results of the present study demonstrate that human CBMC, when co-cultured with irradiated MT-2 cells and maintained in IL-4, were susceptible to transmission and establishment of chronic HTLV-I infection, as were those cultured in the presence of IL-2. Moreover, in contrast to control cells cultured in complete medium in the absence of cytokines, HTLV-I-infected cell lines generated and maintained in the presence of IL-4 showed an initial prolonged phase of profuse growth. However, in contrast to HTLV-I-infected cell lines maintained in IL-2, none of them survived for more than 14 weeks. We were unable to prolong the growth of IL-4-conditioned infected cultures even by mitogen stimulation or re-exposure to MT-2 cells (data not shown). Infected cell lines maintained in IL-4 were capable of binding IL-4, although with decreasing efficiency. This decrease in binding of IL-4 could be one of the reasons why cells grown in IL-4 failed to survive indefinitely in culture. However, IL-4 was unable to support the growth of HTLV-I-infected neonatal cells previously maintained in long-term culture with IL-2, in spite of their ability to bind IL-4. The way in which cells were conditioned at the onset and during the first phase of culture was critical. Interestingly, IL-2 was unable to rescue the cells after they had been grown in IL-4 for some weeks. Conversely, in cases where IL-2 was initially added to the culture together with IL-4 they synergized and the cultures grew better than when maintained in IL-2 alone (data not shown). A wide variability in the capacity of individual infected cultures to grow in the presence of IL-2 or IL-4 was observed. This could obviously be due to the fact that cell growth in our experimental system is the result of many unknown variables, such as individual immune response of effector cells towards virus infection and individual sensitivity to cytokine stimulation of cells from single donors.

It seems clear that recipient cells do not need the presence of IL-2 in order to become infected and to replicate the virus. In fact, the viral message was constantly expressed at high levels in CBMC co-cultured with MT-2 cells and maintained in either IL-2 or IL-4. This is in agreement with recently reported results showing that microcultures from PBMC exposed to HTLV-I in the presence of IL-4 become productively infected, as demonstrated by p24 antigen in culture supernatants (Persaud et al., 1995). These authors have also shown that in the presence of cytokines other than IL-2, i.e. IL-4 and IL-7, HTLV-I-infected clonal populations of T-cells could be propagated in long-term culture, although at a relatively low rate in comparison with cultures kept in IL-2; maximum observation time in culture was not specified. In our hands, neonatal mononuclear cells exposed to HTLV-I could not be propagated in culture beyond the 14th week. Different experimental conditions could account for this discrepancy, such as the use of clonal microcultures from PBMC as target cells or the use of irradiated C91/PL cells as virus donor cells. Cell lines infected with HTLV-I in the presence of IL-4 showed characteristics which clearly differentiated them from those infected in the presence of IL-2. Firstly, addition of IL-4 as a growth factor induces a pattern of cytokine production unlike that which occurs in cultures kept in IL-2. In fact, IL-4 induces upregulation of the IL-4 message in infected cells. Furthermore, a very low level IL-2 message was observed. This also demonstrates that the transient growth of cultures kept in IL-4 was not due to upregulation of the endogenous message for IL-2. Secondly, cultures infected with HTLV-I and grown in IL-4 showed a slightly different phenotype compared with cultures maintained in IL-2. In fact, CD25 and HLA-DR were under-expressed in cultures kept in IL-4 when compared with those grown in IL-2. The failure to express activation markers consistently could be related to the fact that these cultures were not prone to immortalization by the virus. The biological function of IL-4 is regulated by binding its specific receptor, which shares a common γ chain with IL-2 and other cytokine receptors (Kondo et al., 1993). Our results seem to suggest that signal transduction through this common chain of IL-2 receptors either does not function properly in infected cells or is not involved in the immortalization process. In addition, it has recently been demonstrated that IL-2-dependent HTLV-I-infected cultures exhibit an inducible Janus kinase (JAK)-signal transducer and a transcription activation (STAT) pathway that becomes constitutively activated in HTLV-I-transformed cells (Migone et al., 1995; Xu et al., 1995). The JAK/STAT pathway is shared by both IL-2 and IL-4 (Witthuhn et al., 1994). Our results suggest that this pathway might not be activated by IL-4 during HTLV-I infection, or, alternatively, it does not play a central role in the process of HTLV-I-driven transformation. It has recently been documented that mRNA for IL-4 is not constitutively expressed in mononuclear cells from ATLL patients (Mori et al., 1994). Taken together, this observation and our results seem to rule out the possibility that an
autocrine or paracrine loop through IL-4 is involved in late phase transformation in cells infected by HTLV-I. It has been recently proposed that activation of signals able to protect infected cells from death by apoptosis could be a central event in transformation exerted by HTLV-I (Copeland et al., 1994). It is possible that the presence of IL-2, but not IL-4, plays a concurrent role in such a phenomenon. Studies are in progress to investigate this possibility.

This work was supported in part by CNR, Special Projects ‘Cytokines’ and ‘Cell cycle and Apoptosis’, Grant CNR no. 95.01722.CT04 to B. Macchi, and by MURST. We would like to thank Prof. Gianfranco del Prete for his helpful comments on this manuscript. B. Macchi is a participant in the HTLV-I European network (HERN).

References


Ceroni, M., Piccardo, P., Rodgers-Johnson, P., Mora, C., Asher, D., Gajdusek, C. & Gibbs, C. J. (1988). Detection of HTLV type I Tax in transformation exerted by HTLV-I (Copeland et al., 1994). It has been recently proposed that activation of signals able to protect infected cells from death by apoptosis could be a central event in transformation exerted by HTLV-I (Copeland et al., 1994). It is possible that the presence of IL-2, but not IL-4, plays a concurrent role in such a phenomenon. Studies are in progress to investigate this possibility.

This work was supported in part by CNR, Special Projects ‘Cytokines’ and ‘Cell cycle and Apoptosis’, Grant CNR no. 95.01722.CT04 to B. Macchi, and by MURST. We would like to thank Prof. Gianfranco del Prete for his helpful comments on this manuscript. B. Macchi is a participant in the HTLV-I European network (HERN).

References


Received 12 February 1997; Accepted 20 May 1997