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Human Th1 and Th2 T-cell clones are equally susceptible to infection and immortalization by human T-lymphotropic virus type I

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Human CD4 Th1 and Th2 clones were infected with human T-lymphotropic virus type I (HTLV-I) and followed up for a 12 month period in culture. PCR analysis showed that proviral DNA and viral mRNA were present in both Th1 and Th2 infected clones, throughout the entire culture period. Thus, HTLV-I exhibited neither preferential tropism nor exerted differential immortalizing activity in Th1 versus Th2 cells. All the infected clones immediately lost their antigen dependency for growth and continuously proliferated in IL-2-conditioned medium without need for additional stimulation. Infected Th1 and Th2 clones equally showed high expression of CD25, HLA-DR, CD44, CD30 and CD45RO. Infection with HTLV-I altered the cytokine profile in Th1 and Th2 clones. Both types of clones produced IL-6 and TNF-α. Th1 infected clones retained their ability to secrete IFN-γ, but lost IL-2 gene expression. Th2 infected clones lost IL-4 gene expression, retained the ability to produce small amounts of IL-5 and acquired IFN-γ expression.

Mechanisms underlying the pathogenesis of human T-lymphotropic virus type I (HTLV-I) infection have not yet been entirely clarified. Although HTLV-I is associated with disorders affecting different systems and organs (Yoshida *et al.*, 1982; Ceroni *et al.*, 1988; Osame *et al.*, 1986; Sagawa *et al.*, 1995; Nishioka *et al.*, 1989), the effect of the virus at cell level seems to be restricted to the activation and transformation of $CD4^+$ and, less frequently, $CD8^+$ T lymphocytes (Macchi *et al.*, 1987; Richardson *et al.*, 1990, 1997). Activation and proliferation of HTLV-I infected cells could involve deregulation of the check points in the cell cycle (Cereseto *et al.*, 1996), prevention of apoptosis (Copeland *et al.*, 1994), and induction of an autocrine loop involving the IL-2/IL-2Rα chain (Siekevitz *et al.*, 1987). These effects may be triggered by the trans-activating activity of *tax* (Franchini, 1995).

There is increasing evidence to suggest that the immune response against different pathogens involves various effector cells that can be classified into distinct functional populations on the basis of their patterns of cytokine production. Two polarized $CD4^+$ T-cell subpopulations have been identified at clonal level, termed Th1 and Th2. Th1 cells produce IL-2, IFN-γ and TNF-β, whereas Th2 produce IL-4, IL-5, IL-6 and IL-13 (Del Prete *et al.*, 1991). Previous studies have shown that Th1/Th2 cells play different roles in many infectious diseases (Abbas *et al.*, 1996). Regarding human retroviruses, it has been suggested that the Th2 pattern could be associated with the progression of human immunodeficiency virus (HIV) infection. HIV exhibited preferential replication in Th2 cells (Maggi *et al.*, 1994), whereas Th1 cells were associated with an immune response against the virus (Vyakarnam *et al.*, 1995). We have recently shown that human neonatal lymphocytes grown in the presence of IL-4 and showing a Th2-like phenotype could be infected, though not immortalized, by HTLV-I, whereas those grown in IL-2 were stably growing in culture (Mastino *et al.*, 1997). We asked therefore whether Th2 cells could be refractory to the immortalizing activity of HTLV-I. For this purpose, human CD4 clones with a stable Th1 or Th2 profile were infected with HTLV-I *in vitro* and sequentially analysed during a 12 month study.

The three Th1 clones utilized were specific for the PPD antigen (NDP24, NDP16, NDP23), while the three Th2 clones were specific for tetanus toxoid (TT60, TT2) or *Toxocara canis*

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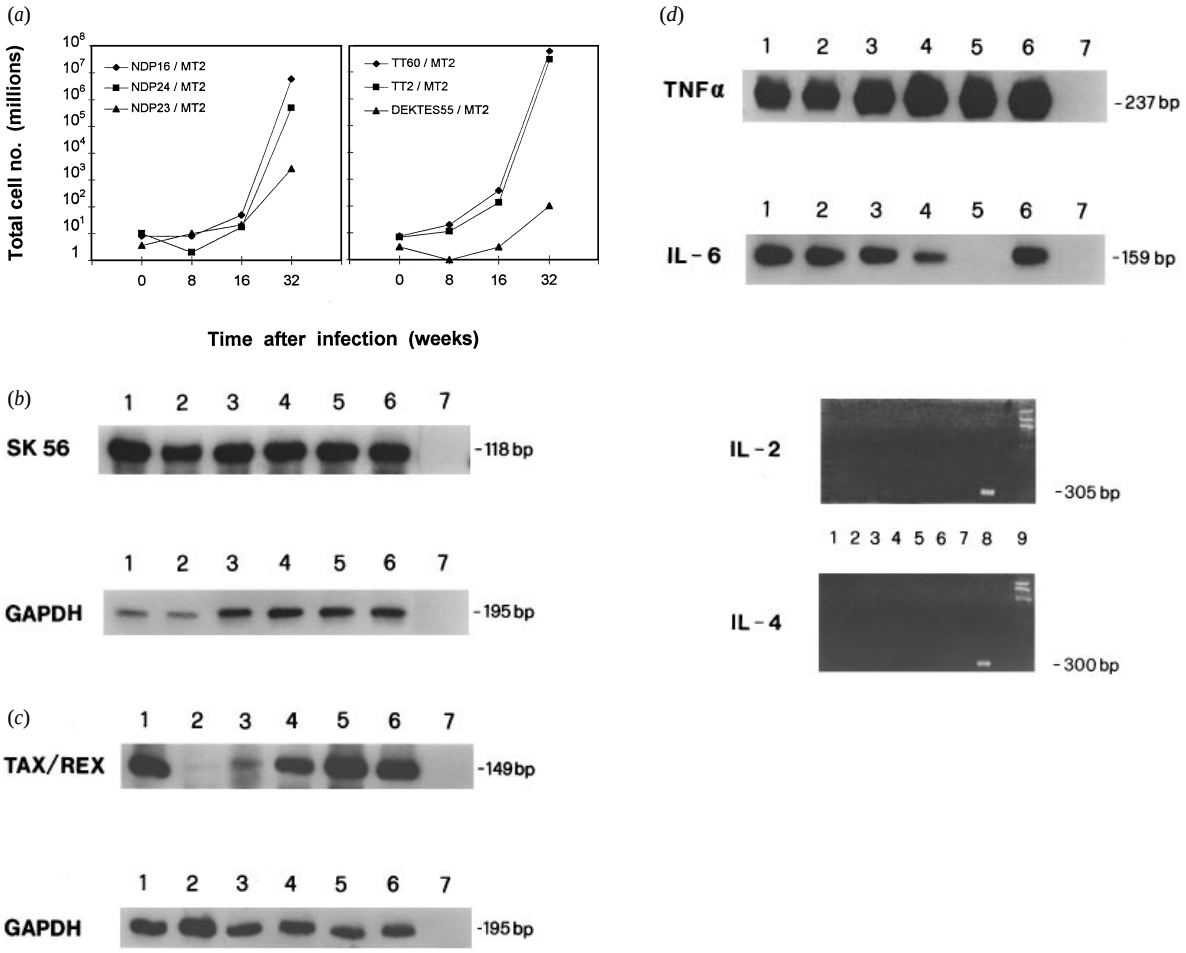

Fig. 1. (*a*) Growth curve of human Th1 (NDP16/MT2, NDP24/MT2, NDP23/MT2) and Th2 (TT60/MT2, TT2/MT2, DEKTES55/MT2) generated *in vitro* and maintained in culture, in the presence of IL-2, by repeated cycles of stimulation with specific antigens and then infected with HTLV-I by cocultivation with lethally irradiated MT2 virus-donor cells. After infection, Th1 and Th2 cell clones, designated as XXX/MT2, where XXX is the code of the corresponding uninfected clones, were cultured in the presence of IL-2 without any kind of antigen-specific or non-specific stimulation, and cell growth was monitored by evaluating viability using the trypan blue dye exclusion test. (*b*) Presence of HTLV-I DNA in the same Th1 and Th2 clones infected *in vitro* with HTLV-I as reported in (*a*), as detected by DNA PCR of the HTLV-I *pol* region (SK56), 8 months post-infection. GAPDH, internal control. Lanes 1, NDP24/MT2; 2, NDP23/MT2; 3, NDP16/MT2; 4, TT2/MT2; 5, TT60/MT2; 6, DEKTES55/MT2; and 7, PCR mix. (*c*) Expression of HTLV-I viral mRNA in the same Th1 and Th2 clones infected *in vitro* with HTLV-I as reported in (*a*), as detected by RT–PCR of HTLV-I *tax*/*rex*, 8 months post-infection. GAPDH, internal control. Lanes 1, NDP24/MT2; 2, NDP23/MT2; 3, NDP16/MT2; 4, TT2/MT2; 5, TT60/MT2; 6, DEKTES55/MT2; and 7, PCR mix. (*d*) Expression of cytokine mRNAs in the same Th1 and Th2 clones infected *in vitro* with HTLV-I as reported in (*a*), as detected by non-quantitative RT–PCR of TNF-α, IL-6, IL-2 and IL-4, 8 months post-infection. Lanes 1, NDP24/MT2; 2, NDP23/MT2; 3, NDP16/MT2; 4, TT2/MT2; 5, TT60/MT2; 6, DEKTES55/MT2; 7, PCR mix; 8, positive control (PBMC stimulated with PHA at 2 μg/ml for 16 h); and 9, molecular mass markers, ϕX174 RF DNA/*Hae*III digest. Expression of TNF-α and IL-6 mRNAs was revealed by liquid hybridization, while the expression of IL-2 and IL-4 mRNAs was visualized by running onto a 1% agarose gel in TBE containing ethidium bromide.

(DEKTES55) antigens. They were generated from PBMC and maintained in culture, by means of repeated cycles of stimulation with the specific antigen until infection, as previously described (Del Prete *et al.*, 1991). For infection, T-cell clones, previously stimulated with 0·5 μg/ml PHA for 48 h, were cocultured with lethally irradiated (120 Gy, from a caesium gamma cell 1000, Atomic Energy of Canada), MT2 cells at an acceptor : donor ratio of 5 : 1 (Miyoshi *et al.*, 1981). Human recombinant IL-2 (Cetus) was added to the cultures at a final concentration of 20 U/ml. Cultures were passaged weekly, for 12 months post-infection. Immediately after virus transmission, all Th1 and Th2 clones could be maintained in culture in the presence of IL-2, without any kind of antigen-specific or non-specific stimulation. At each cell passage, cell growth was monitored by evaluating living cells using the trypan blue dye exclusion test and the cell concentration was readjusted to 1×10^6/ml as previously described (Mastino *et al.*, 1997). The growth rate, while exhibiting individual

variability, did not show a different pattern in Th1 or Th2 infected clones. Starting 16 weeks after infection a rapid enhancement of the doubling time occurred in all the clones, except in the DEKTES55/MT2 clone in which it was delayed (Fig. 1*a*). The Th1 and Th2 infected clones were assayed for the presence and expression of the virus. Analysis of proviral DNA by DNA PCR was performed as previously described (Macchi *et al.*, 1997). Samples were prepared by DNA extraction through incubation with proteinase K at 37 °C and further extraction in phenol–chloroform–isoamyl alcohol (50:49:1). Amplification by PCR of 1 μg DNA was performed in a 50 μl mix containing 1 × PCR buffer, 0·2 mM dNTPs (Pharmacia Biotech), 0·5 μM primer pair specific for the Pol region (SK54, SK55; Perkin Elmer) of HTLV-I, or for GAPDH as internal control (Genis *et al.*, 1992), and 2·5 U *Taq* polymerase (Boehringer Mannheim). Samples were subjected to 40 cycles of PCR amplification, each cycle consisting of 30 s at 94 °C (DNA denaturation), 30 s at 55 °C (primer annealing) and 45 s at 72 °C (primer extension) in a DNA thermal cycler 2400 (Perkin Elmer). Following the final cycle, samples were incubated at 72 °C for 7 min to ensure the completion of the final extension step.

Fig. 1(*b*) illustrates an assay performed 8 months post-infection and shows that HTLV-I proviral DNA was present in both Th1 and Th2 infected/immortalized clones. Total RNA was extracted from 5×10^6 cells previously washed in PBS. Isolation of RNA was performed using a commercial kit (RNAfast-II, Molecular Bio-products) according to the manufacturer's instructions. Total RNA was then reverse transcribed into cDNA in 25 μl reaction mix as follows: 2 μg RNA 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 Mu-MLV RT (New England Biolabs) for 1 h at 37 °C. The reaction mix was incubated at 95 °C for 5 min in order to inactivate RT and then it was chilled on ice. Three μl of the cDNA was amplified by PCR in a total volume of 50 μl. Amplifications with RPX3 and RPX4 primers (Kinoshita *et al.*, 1989) were performed for 40 cycles using the amplification program described above for SK54 and SK55. RPX3 and RPX4 primers specifically recognize the Tax/Rex region of HTLV-I. The Tax/Rex region is expressed as a doubly spliced mRNA, and RPX3 and RPX4 primers are located upstream and downstream, respectively, of the second junction site for Tax/Rex mRNA. Viral mRNA was expressed in both Th1 and Th2 clones, albeit at a lower level in the clone NDP23/MT2 (Fig. 1*c*). Amplified DNAs were analysed by liquid hybridization and samples were probed using specific ^{32}P-end-labelled oligonucleotides as described previously (Mastino *et al.*, 1997).

Infected clones were studied to assess whether they maintained the original pattern of cytokine production typical of Th1 or Th2 phenotype. Amplifications of cDNA for IL-2, IL-4, IL-6 and TNF-α mRNA detection by non-quantitative RT–PCR analysis (Mastino *et al.*, 1997) were performed as described above. The samples were also amplified with primers specific for GAPDH as an internal control. Both Th1 and Th2 HTLV-I infected clones expressed mRNA for TNF-α (Fig. 1*d*). Similarly, IL-6 was expressed in all the infected clones, except for TT60. It has been suggested that IL-6 and TNF-α might regulate spontaneous lymphocyte proliferation in HTLVI/II infected individuals (Lal & Rudolph, 1991), although recent data argue against a role for IL-6 in virus-induced cell proliferation (Richardson *et al.*, 1997). Conversely, analysis of mRNA for IL-2 and IL-4 showed that Th1 and Th2 clones lost their ability to express IL-2 and IL-4, respectively. The measurement of cytokines released in culture supernatants, assessed by commercially available ELISA kits, according to the manufacturer's instructions, showed that not only Th1, but also Th2 clones released IFN-γ following HTLV-I infection (Table 1). In addition, TNF-α was equally released in culture supernatants by both Th1 and Th2 clones. Following HTLV-I immortalization, Th2 clones still produced IL-5, though at a lower level. Thus, a change in cytokine expression was a common feature in HTLV-I infected Th1 and Th2 clones. However, this change occurred differently from that occurring *in vitro* in herpes saimiri infection, where Th1 clones retained their original phenotype, while Th2 clones expressed a Th0 phenotype (De Carli *et al.*, 1993). This suggests specificity in the HTLV-I-driven pattern of cytokine production, which seems to be largely not dependent on the original pattern of cytokine production in either Th1 or Th2 clones. Interestingly, data obtained from the analysis of leukaemic cells from adult T-cell leukaemia/lymphoma patients have shown that the pattern of cytokine production was heterogeneous and could be ascribed neither to the Th1 nor to the Th2 pattern (Noma *et al.*, 1989; Richardson *et al.*, 1997). The results reported in Table 2 show that both Th1 and Th2 HTLV-I infected clones were proliferating spontaneously, when assayed 8 months post-infection. Cell proliferation was assessed by [^{3}H]thymidine

Table 1. Cytokine production by HTLV-I infected Th1 and Th2 clones

Release of cytokines in culture supernatants was assessed using commercial ELISA kits 8 months after infection.

Clone	IFN-γ (U/ml)	IL-5 (U/ml)	TNF-α (pg/ml)
NDP24/MT2	17	< 0·4	ND
NDP16/MT2	22	< 0·4	190
NDP23/MT2	120	< 0·4	1550
TT60/MT2	37	0·8	340
TT2/MT2	53	0·6	190
DEKTES55/MT2	16	4·0	ND

ND, Not done.

Table 2. [^{3}H]Thymidine incorporation of HTLV-I infected Th1 and Th2 clones in the presence of different stimuli

Clone*	Medium	Irradiated allogenic PBMC	PHA (2μg/ml)
NDP24/MT2	52564 ± 3630	16920 ± 1301	44953 ± 4492
NDP16/MT2	148500 ± 16543	62001 ± 7959	67704 ± 991
NDP23/MT2	46494 ± 7885	30045 ± 902	47700 ± 3746
TT60/MT2	119000 ± 3830	28927 ± 722	47930 ± 4794
TT2/MT2	64745 ± 3370	46444 ± 3502	63910 ± 5616
DEKTES55/MT2	23568 ± 1863	13529 ± 621	42704 ± 3535
MT2	488250 ± 13574	ND	ND
PBMC	4247 ± 786	30000 ± 635	289750 ± 17017

* Clones were distributed at 1×10^5 cells per well in 96-well plates and maintained in IL-2-free medium or stimulated with irradiated allogenic donor cells at a responder:stimulator ratio of 1:5 or with PHA for 48 h. MT2 is a chronically HTLV-I infected T-cell line. PBMC are peripheral blood mononuclear cells from a healthy donor.
ND, Not done.

incorporation. Briefly, infected clones were washed and resuspended in IL-2-free medium. Part of the cells were then stimulated with 5 μg/ml PHA, or with irradiated allogenic PBMC, for 48–72 h in 96-well plates at 1×10^5 cells per well. Then, 1 μCi per well [^{3}H]thymidine (Amersham) was added and cultures were harvested following overnight incubation. As opposed to uninfected fresh PBMC, neither Th1 nor Th2 infected clones responded to allogenic or PHA stimulation, suggesting progression toward immortalization. Surface molecule analysis was performed using specific monoclonal antibodies (MAbs) and flow cytometry, as previously described (Mastino *et al*., 1997). The following mouse anti-human MAbs were used: phycoerythrin-conjugated CD4, CD3, CD25 and CD45RO, and fluorescein-conjugated CD8, CD30, HLA-DR, CD44 and CD45RA (Becton-Dickinson). For double fluorescence labelling, MAbs were combined as follows: CD4/CD8, CD25/HLA-DR, CD45RO/CD45RA, CD3/CD44 and CD4/CD30. Both Th1 and Th2 infected clones showed a phenotype typical of activated cells since they expressed CD25 and HLA-DR molecules. In addition, Th1 and Th2 clones were positive for CD44 and CD45RO (data not shown). Our results are consistent with those of Prince *et al*. (1995) who showed that most spontaneously proliferating T lymphocytes in HTLV-I infected individuals were CD45RO$^+$. Moreover, the CD30 molecule, usually poorly (or not) expressed on Th1 clones (Del Prete *et al*., 1995), was present on HTLV-I infected Th1 clones as well (data not shown).

In this study we have shown that HTLV-I is able to replicate in Th2 as efficiently as in Th1 human clones. Furthermore, it has to be pointed out that in Th2 infected clones IL-4 expression progressively decreased and finally stopped completely. Likewise, expression of IL-2 was inhibited in HTLV-I infected IL-2 dependent or independent tumour cell lines (Arya *et al*., 1984). Taken together, our results outline two important aspects of HTLV-I infection. First, the pattern of endogenous cytokine production of target cells does not influence susceptibility to immortalization. Furthermore, the virus modifies the characteristics of infected cells, resulting in the selection of a virus-specific heterogeneous Th phenotype of immortalized cells, independent of their original phenotype. Secondly, it is intriguing that efficient infection and immortalization of Th2 clones seems to limit the possible role played by an IL-2/IL-2R autocrine loop in the transforming activity of HTLV-I, as suggested by some studies (Tendler *et al*., 1990). Our data on Th2 clones are compatible with previous reports, suggesting that spontaneous proliferation of lymphocytes from HTLV-I-associated myelopathy patients was not affected by the inhibition of IL-2-mediated signals (Hollsberg *et al*., 1992). However, our data do not rule out that growth factors and their receptors could play a pivotal role during HTLV-I-driven transformation of lymphocytes. One simple hypothesis is that during immortalization paracrine factors produced in the microenviroment are more important than those endogenously produced by infected cells. This hypothesis could explain, at least in part, why cell lines from human cord blood lymphocytes, cultured using IL-4 as growth factor and showing a Th2-like phenotype, could be infected by HTLV-I *in vitro* but not immortalized (Mastino *et al*., 1997), which is different to Th2 clones grown in IL-2 as reported in the present work. Another hypothesis is that factors other than those examined, including IL-7 as suggested by *in vitro* experiments (Persaud *et al*., 1995), could play a role in an autocrine/paracrine loop during infection. A growth factor/growth factor receptor loop could act as inhibitory signals for cell death, possibly interacting with apoptosis processes. These phenomena may play important roles in the early stages of leukaemogenesis and further

information on their occurrence could greatly contribute to elucidating the oncogenetic property of HTLV-I.

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