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MONONUCLEAR CELLS FROM PERIPHERAL BLOOD OF ADULT DONORS AND FROM CORD BLOOD ARE EQUALLY PROTECTED BY α- AND β-INTERFERONS AGAINST INFECTION WITH HTLV-I

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SUMMARY

Human mononuclear cells derived from peripheral blood of adult donors (PBMC) or from neonatal cord blood (CBMC) were found to be equally sensitive to the protective effect of α- and β-interferons (IFNs) against the infection with HTLV-I during long-term culture. The effect of IFNs was evidenced by a remarkable reduction of the number of virus-positive cells during culture as evaluated by indirect immunofluorescence for the p19 virus core protein. Moreover, the appearance of p19-positive immortalized clones was inhibited by IFNs in PBMC co-cultures, whereas it was delayed in CBMC cultures. These kinetics are in relation with the higher permissivity of CBMC to the virus in comparison with PBMC, since in CBMC cultures infected cells can be clearly detected starting already 1 week post-infection (p.i.), whereas in PBMC cultures their appearance time is approximately at the 6th week p.i.

IFNs acted by 'priming' PBMC and CBMC to an active antiviral competence, since one single treatment with 1000 IU/ml of α- or β-IFN at the onset of the co-culture of mononuclear cells with irradiated virus-donor cells was able to maintain very low levels of infection for approximately 6 weeks in CBMC cultures and at least for 18 weeks in PBMC cultures. As a consequence, it seems likely that IFN action is mediated by the expression of a defined, although not completely identified, set of genes in the host cells.

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INTRODUCTION

HTLV-I (human T-cell leukaemia virus type I) is a known human retrovirus capable of transforming CD4 + lymphocytes both *in vivo* and *in vitro*, thus inducing the clinical state of an adult T-cell leukaemia (ATL) [1–3]. Since HTLV-I-induced transformation is targeted to an immunocompetent effector cell prevalently with CD4 + 'helper/inducer' phenotype [4, 5], it follows that host's antiviral immune responses would be directly impaired by virus infection. This fact contributes to the development of a latent, persistent infection, that would be converted into a manifest leukaemia when the immune surveillance falls below a definite threshold. To this end α-, β- and γ-interferons (IFN) showed a remarkable protective effect against HTLV-I infection in human mononuclear cells derived from cord blood (CBMC) [6–9]. This effect was dependent on a direct inhibition of viral replication [6, 7] and on boosting of cell-mediated cytotoxicity of CBMC [8, 9]. All three types of IFNs were effective, although to a different extent. Moreover, they all 'primed' CBMC for a long-lasting antiviral competence, suggesting the involvement of a defined, although mostly unknown, group of cellular genes. There is experimental evidence that expression of some growth factors like IL-1β [10, 11], IL-2 [12] and c-sis/PDGF2 [11] is affected by HTLV-I and differently modulated in IFN-treated CBMC co-cultures [10, 11]. These experiments provided a rationale for an experimental therapy with IFNs for prevention of mother-to-fetus transmission and could represent a model for *in vitro* infection of immature mononuclear cells, that might, at least in part, mimic the infection of bone marrow precursors. However, in view of a possible therapeutical use of IFNs for prevention of HTLV-I infection in adults at risk and/or prevention of ATL development in seropositive individuals, the question remained to be answered, whether mature immunocompetent virus-target cells, like mononuclear cells derived from peripheral blood of adult donors, would also be susceptible to the protective effect of IFNs.

α- and β-IFN were preferentially tested, since they were found to be more active than γ-IFN in the overall protection against HTLV-I infection [6–9]. In the experiments here described, mononuclear cells from adult donors compared with cells derived from umbilical blood under the same experimental conditions, were equally protected from HTLV-I infection by 'priming' with α- and β-IFN at time 0. These data provide additional support to a therapeutical approach with IFNs in the early phase of HTLV-I infection by contributing experimental evidence about the cellular mechanisms underlying the possible remission of ATL following treatment with β-IFN, as recently described [13].

MATERIALS AND METHODS

Cell cultures and infection

Human mononuclear cells were isolated from peripheral blood of adult donors (PBMC) or from umbilical cord blood (CBMC) by Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden) under routine procedure and cultured in 25 cm^2 flasks (Falcon, Oxnard, USA) in RPMI 1640 medium (Gibco, Grand Island, USA), supplemented with 20% fetal calf serum (Gibco), 2 mM glutamine (Gibco), 100 IU/

ml penicillin/streptomycin (Gibco). The culture medium was additionally supplemented with 20–40 IU/ml of recombinant interleukin-2 (IL-2, kindly provided by Hoffman-La Roche, Basel, Switzerland), to allow the long-term survival of lymphocytes.

MT-2 cells, an HTLV-I producing, cord blood-derived, established cell line [4], were also grown in supplemented RPMI 1640 medium, in the absence of IL-2, and diluted serially twice a week.

Infection with HTLV-I was obtained by co-culturing freshly isolated mononuclear cells with lethally irradiated MT-2 cells at a ratio of 5:1 [14], CBMC/MT-2 cultures were maintained in culture by addition of 20 IU/ml of recombinant IL-2 to supplemented RPMI 1640 medium, whereas for PBMC/MT-2 co-cultures 40 IU/ml IL-2 were required to allow the long-term survival of lymphocytes. The culture medium was renewed every week and fresh IL-2 was added. The co-cultures were not diluted until the concentration of cells reached 10^6/ml (around 8 weeks post-infection, p.i.).

Irradiation of MT-2 cells in vitro

MT-2 cells were irradiated *in vitro* with 12 000 rads using a caesium-137 irradiator (Gamma cell 1000, model A, A.E.C.L., Canada) delivering γ-rays at the rate of 1000 rads/min, suspended in culture medium in 50 ml tubes at the concentration of 10^6 cells/ml. Cells were then washed twice, resuspended in the culture medium and kept at 4°C until used.

Interferon treatment

Recombinant α_2-IFN was kindly provided by Hoffman-La Roche and purified natural β-IFN by Sclavo (Siena, Italy). IFNs were diluted in RPMI 1640 medium plus 5% fetal calf serum, at the concentration of 20 000 IU/ml and aliquots were stored at − 70°C.

For treatment of mononuclear cells, IFNs were used at the concentration of 1000 IU/ml and added to mononuclear cells/MT-2 co-cultures only at the onset of the co-culture (time 0), in line with our previous reports on 'priming' of CBMC by α- and β-IFN to a cell specific long-lasting antiviral competence [6–9].

Time course of HTLV-I infection in PBMC and CBMC co-cultures

Infection was evaluated by indirect immunofluorescence for the p19 viral core protein [15] on methanol/acetone (1:3) fixed samples. An average of 500 cells was scored for each sample by fluorescence microscopy (Leitz, Wetzelar, FRG) and the number of per cent positive cells per sample was compared by χ^2 analysis. The amount of integrated provirus sequences in genomic DNA of infected PBMC and CBMC co-cultures was evaluated by dot blot analysis, as previously described [8]. Briefly, genomic DNA was extracted by standard proteinase K method and samples, previously denatured and then neutralized in 2 M ammonium acetate, were spotted on nitrocellulose filter (Schleicher & Schüll, Dassel, FRG) and hybridized with the Sst 1–Sst 1 fragment of HTLV-I (approximately 8·5 kb, accounting for almost the entire virus genome), digested from pMT-2 plasmid

(kindly given by R.C. Gallo) and labelled with ^{32}P-ATP by standard nick translation procedure. After hybridization, filters were exposed for autoradiography (Kodak XAR-5 films, Kodak Co., Rochester, USA).

Proliferation assay in mixed lymphocyte/tumour cell cultures (MLTC) and clonal expansion of infected mononuclear cells

Growth rate of co-cultures (i.e. MLTC mononuclear cells reaction against the allogeneic MT-2 infecting cells) was evaluated by direct counting of viable cells and by [^{3}H]thymidine incorporation during the 1st week p.i. [Methyl]-[^{3}H]thymidine was purchased by Amersham Int. (Amersham, UK). 2×10^5 mononuclear cells/well were plated on day 0 in 96-microtitre plates (Falcon) and co-cultured with 4×10^4 irradiated MT-2 cells, in IL-2 enriched medium.

One μCi of thymidine/well was added on day 0, 2, 4, 6, 8, 10 and cells were harvested 18 h later by microtitre cell harvester (Titerteck 530, Flow Lab., Irvine, UK). The incorporated radioactivity was measured by a scintillation beta-counter (LKB, Bromma, Sweden) and mean c.p.m. ± standard error (SE) of quadruplicate samples was calculated.

The [^{3}H]thymidine incorporation of mononuclear cells/MT-2 co-cultures was further monitored weekly until 8 weeks of culture under the conditions above described, and data were compared with direct count of viable cell number. In these kinetics, data are to be considered as related to the proliferation of mononuclear cells during the autologous transmission phase that follows the initial allogenic transmission and later on during the phase of expansion of virus-infected cells.

RESULTS

Cell growth and proliferation rate of virus-infected PBMC/MT-2 and CBMC/MT-2 co-cultures

PBMC and CBMC were co-cultivated *in vitro* with the HTLV-I donor cell line, MT-2, at 5:1 acceptor to donor cells ratio, with or without 1000 IU/ml of α- or β-IFN. The growth curves of PBMC/MT-2 and CBMC/MT-2 co-cultures through 8 weeks of culture are shown in Table I.

At day 0 the acceptor cells were plated at 1×10^6 cells/ml. One week later, the number of co-cultured PBMC and CBMC cells decreased to approximately half concentration of that of plated cells, both in the presence or absence of α- or β-IFN. Starting at this point, the growth curves of infected PBMC and CBMC partially diverged, since the cell number of co-cultured PBMC decreased progressively during the first 8 weeks of culture, independently of IFN supply. After this time, the co-cultures reached a stage in which the number of cells remained constant through months (data not shown). On the other hand, the CBMC/MT-2 co-cultures showed a decreased number of viable cells throughout the first 3 weeks p.i., but later the cell number returned to the initial value. At this stage, α-IFN delayed the clonal expansion of infected CBMC, whereas β-IFN hardly affected it [8]. As shown for co-cultured PBMC or CBMC, cell viability of normal PBMC and CBMC not exposed to HTLV-I, routinely maintained in IL-2

Table I

Cell growth of PBMC and CBMC co-cultured with irradiated MT-2 cells and monitored during culture time

Sample	Viable cells × 10^5/ml Week							
	0	1	2	3	4	5	6	8
PBMC/MT-2	10	4·2±0·5	5·5±0·3	8·0±0·3	2·0±0·2	1·5±0·4	6·0±0·3	3·5±0·5
PBMC/MT-2 + α-IFN	10	4·6±0·4	3·8±0·2*	6·0±0·3**	1·0±0·3**	1·0±0·2	3·0±0·2**	4·2±0·3
PBMC/MT-2 + β-IFN	10	5·0±0·1*	6·5±0·2**	6·7±0·1**	NT	NT	4·0±0·4**	3·0±0·2
CBMC/MT-2	10	3·4±0·2	4·2±0·2	6·4±0·3	5·5±0·4	9·2±0·2	9·8±0·3	NT
CBMC/MT-2 + α-IFN	10	5·2±0·4**	4·6±0·3	4·4±0·2***	6·1±0·3	4·1±0·5***	6·3±0·3***	NT
CBMC/MT-2 + β-IFN	10	5·8±0·5**	5·9±0·2***	4·0±0·2***	10·0±0·3***	8·0±0·3**	6·0±0·2***	NT

Non-infected control cultures of PBMC survived until 10 weeks p.i., although the cell number was decreased from 1×10^6/ml cells plated on day 0 to approximately zero. A similar survival pattern was observed with non-infected CBMC, although they approached zero at earlier time, around 6 weeks p.i.; each value represents the arithmetic mean obtained from triplicate cultures ± SD.

Statistical analysis by Student's 2-sided t-test with respect to the corresponding untreated controls.

*$P<0·05$; **$P<0·01$, ***$P<0·001$.

enriched medium, was unchanged after addition of either α- or β-IFN (1000 IU/ml) to culture medium, until 5 weeks of culture. Although α- and β-IFN inhibited the [³H]thymidine incorporation in normal CBMC cultures grown for 1 week in IL-2 supplemented medium, they did not affect the low incorporation rate of CBMC/MT-2 co-cultures [8]. To further analyse the proliferation rate of both PBMC and CBMC co-cultures in long-term culture in the presence of α- or β-IFN, a [³H]thymidine incorporation assay was performed until the 8th week of co-culture. PBMC/MT-2 and CBMC/MT-2 co-cultures were also grown in IL-2 enriched medium, with or without 1000 IU/ml of α- or β-IFN. The patterns of thymidine incorporation showed that the proliferative response of PBMC to HTLV-I, irrespective of the addition of IFNs, was biphasic, since it reached a peak 1 week after the onset of the co-culture and progressively decreased throughout the following 6 weeks, with a transient second peak 3 weeks p.i. (Fig. 1). Six weeks p.i. co-cultured PBMC showed a slight positivity for the p19 virus core protein (see below). On the other hand, the proliferative response of CBMC to HTLV-I seemed to have a different pattern (Fig. 2). Ten days p.i. the proliferation rate of co-cultured cells peaked from the minimal baseline value to a fourfold increase, which remained constant until the 4th week and then declined. IFN-treated co-cultures showed a slightly lower peak but retained a well detectable proliferative capacity until the 8th week of culture. After this time, depending on different donors, infected CBMC could enter in a steady state of survival lasting a few weeks or died within 8 weeks p.i. Immortalized clones, when spontaneously or intentionally selected, arose from CBMC at this stage of infection.
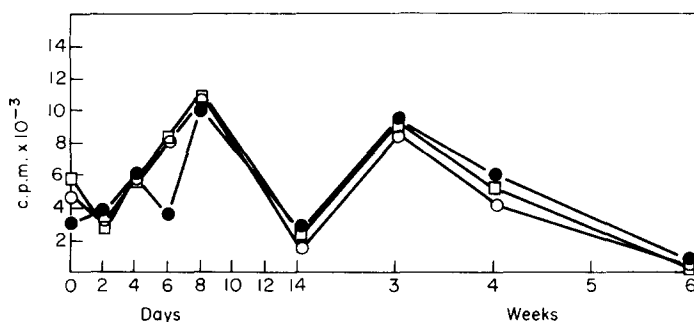

Fig. 1. Time course of [³H]thymidine incorporation in a representative experiment: ● PBMC/MT-2, ○ PBMC/MT-2 + α-IFN 1000 IU/ml, □ PBMC/MT-2 + β-IFN 1000 IU/ml.

Time course of virus infection in PBMC and CBMC co-cultures

The degree of infection of PBMC or CBMC during 8 weeks p.i., monitored as the number of cells expressing the virus core protein p19, showed that HTLV-I infection progressed differently in PBMC as compared to CBMC (Fig. 3).

PBMC/MT-2, as already mentioned, began to express p19 6–10 weeks after the onset of the co-cultures and the percentage of the positive cells increased very
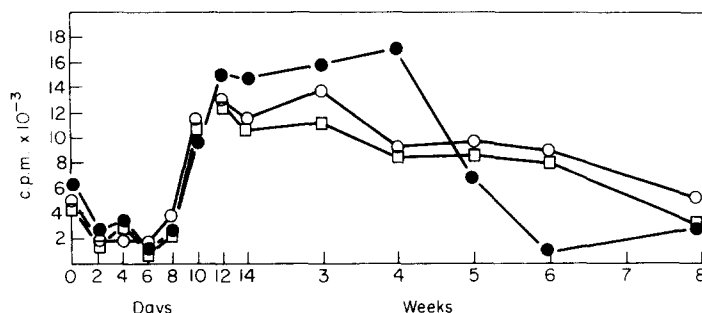
Fig. 2. Time course of [³H]thymidine incorporation in a representative CBMC/MT-2 co-culture: ● CBMC/MT-2; ○ CBMC/MT-2 + α-IFN 1000 IU/ml; □ CBMC/MT-2 + β-IFN 1000 IU/ml.
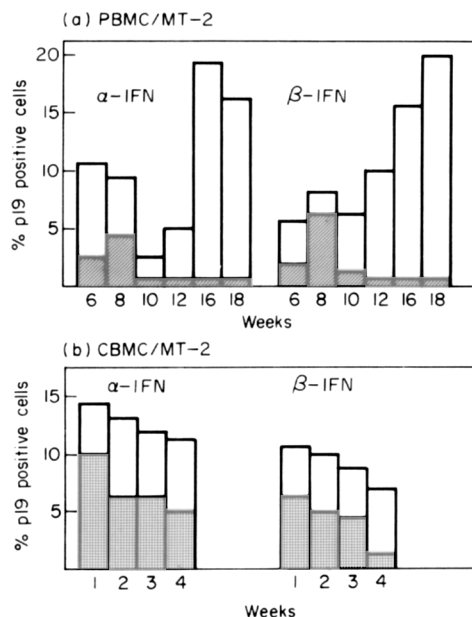

Fig. 3. Time course of p19 expression in PBMC/MT-2 (panel a) or CBMC/MT-2 (panel b) co-cultures. □ untreated cultures; ▨ IFN treated PBMC/MT-2; ▩ IFN treated CBMC/MT-2.

slowly until the 18th week of culture. At this time the cells did not proliferate and the number of viable cells remained unchanged for months. In this late phase PBMC were still IL-2 dependent and morphologically resembled other established HTLV-I positive lines, including MT-2 cells, which grow in clumps. In the absence of α- or β-IFN, PBMC showed a minimal positivity for p19 throughout 10 weeks. Later the percentage of p19 positive cells increased, reaching appreciable levels at 16–18 weeks p.i., and integrated provirus sequences were detectable by dot blot analysis (Fig. 4). In contrast in IFN-treated co-cultures both p19 expression and the amount of integrated provirus were clearly reduced (Figs 3 and 4), becoming predominantly undetectable over 18 weeks of culture.

Infected CBMC expressed the p19 virus core protein much earlier than PBMC. One to two weeks after the onset of the co-culture, CBMC became positive for p19 (Fig. 3) and integrated provirus sequences were detected by dot blots (Fig. 4). The number of positive cells decreased throughout 6 weeks, and later increased again, when the number of infected cells became prevalent, in comparison with non-infected, progressively dying CBMC. Treatment of CBMC co-cultures with α- or β-IFN at time 0 resulted, on average, in a 60% inhibition of p19 expression in terms of percentage of positive cells (Fig. 3), and amount of integrated HTLV-I provirus (Fig. 4). However, after the 6th week of co-culture the few virus-positive cells maintained a growth advantage compared to the non-infected CBMC and progressively emerged (data not shown). Moreover, preliminary experiments showed that the CD4 + subset, separated from CBMC by a monoclonal anti-CD4 antibody conjugated to immunomagnetic beads, were well protected by treatment with β-IFN, resulting in 90% of inhibition of p19 expression as compared to untreated CD4/MT-2 co-cultures, 1 week p.i. Conversely, protection of the CD8 + subset, which is less susceptible to HTLV-I infection, was achieved at a later stage of infection, showing an 80% inhibition with respect to untreated CD8 + /MT-2 co-cultures, 4–5 weeks p.i.
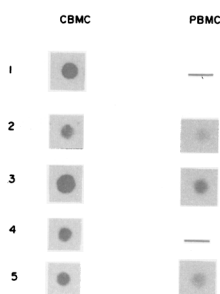

Fig. 4. Dot blot analysis on genomic DNA of co-cultured PBMC or CBMC, extracted 12 weeks p.i. and 2 weeks p.i., respectively. α- or β-IFN (1000 IU/ml) were added only at the onset of the co-culture. 1, MT-2 (HTLV-I +) positive control; 2, PBMC or CBMC negative control; 3, PBMC/MT-2 or CBMC/MT-2 co-cultures; 4, α-IFN-treated CBMC co-culture; 5, β-IFN-treated PBMC or CBMC co-cultures.

It has to be emphasized that the great majority of irradiated MT-2 cells did not survive longer than 4–6 days under standard co-culture conditions, as morphologically evaluated by fluorescence microscopy. Moreover, their viability, when cultured in the absence of immune effector cells, i.e. PBMC or CBMC, was decreased 20-fold, 10 days p.i., thus excluding a significant interference of MT-2 cells in the evaluation of p19 positive mononuclear cells during the culture.

DISCUSSION

Any therapeutic approach for prevention of ATL in endemic areas requires a detailed knowledge of the single steps of virus infection, from allogeneic to

autologous virus transmission and subsequent proliferation of virus-positive cells, that disseminate the virus in the host. Infection with HTLV-I can be easily reproduced under experimental conditions [4] using mononuclear cells from healthy donors as recipient cells and simulating an allogeneic transmission by infecting with the lethally irradiated virus donor cells. This provided an easy and reproducible tool to set up an experimental approach to study retrovirus-induced leukaemia in a human model.

The immune competence of the recipient cell profoundly influences the degree of infection and its kinetics during early weeks p.i. Therefore we compared the susceptibility of both mononuclear cells derived from an adult immunocompetent host, i.e. PBMC, and from neonatal umbilical blood, i.e. CBMC. The use of CBMC as virus-recipient cells would mimic mother-to-fetus transmission and probably can offer additional information on the more generally focused problem of infection of immature, precursor cells (e.g. bone marrow cells, [16]). The higher susceptibility of CBMC to HTLV-I infection, when compared to that of PBMC, is likely to depend mostly on their higher content of proliferating immature T-cells [17, 18]. Conversely, PBMC subpopulations express phenotype markers of mature and scarcely proliferating immunocompetent cells [17, 19, 20]. Actually, although HTLV-I was strongly cytopathic for both PBMC and CBMC co-cultures, as shown by the growth curves and [^{3}H]thymidine incorporation, CBMC showed higher proliferative capacity in comparison with PBMC, especially in a late phase of infection.

In previous reports [6, 9] we had shown that α- and β-IFN had a protective effect against HTLV-I infection of CBMC. It was consequently suggestive to compare CBMC versus PBMC, because of the possibility of defining an immunopharmacological approach for prevention of HTLV-I related disease. Data herein described confirm that α- and β-IFN can exert an antiviral protective effect also in the PBMC model. IFNs had no substantial antiproliferative effect on the early phase of response of both types of mononuclear cells to the virus, i.e. 1–2 weeks p.i. In the following weeks slight proliferative effects of α- and β-IFNs were evidenced in CBMC co-cultures. However, in a later phase, the expansion of virus-positive clones was delayed much more in α-IFN-treated co-cultures as compared to β-IFN [8]. In contrast, PBMC showed an identical pattern of [^{3}H]thymidine incorporation, independently of IFN treatment, until at least 10–12 weeks of culture.

The most remarkable effect of α- and β-IFN was their capacity of keeping the degree of infection at a very low level in both PBMC and CBMC co-cultures. This protective effect was long-lasting and relied on 'priming' of mononuclear cells to an effective antiviral competence, since one single treatment with IFNs at time 0 was able to maintain its effect for approximately 6 weeks in CBMC and 16 weeks in PBMC cultures, which did not survive over this time, as the non-infected control cultures. The fact that the virus core protein p19 appeared in PBMC at a later stage from the onset of the co-culture than in CBMC is probably related to the higher immunocompetence of the former cells and to their higher differentiation stage [17].

On the basis of the results obtained both in PBMC and in CBMC models, it seems that virus expression is initially linked to a non-proliferative phase of the

culture. Preliminary experiments suggested indeed that one of the pathogenic mechanisms of HTLV-I might be dependent on a direct viral influence on the cell cycle of recipient cells, since the mitotic phase is highly permissive for viral integration while a quiescent phase is compatible with transcription and assembly of new virions. In fact, the expression of mitogenic factors like IL-1β [10, 11], IL-2 [12] and c-sic/PDGF/2 [11] is altered in the 1st week p.i., when the proliferative response of CBMC to the allogenic MT-2 infecting cells is suppressed. Data obtained in β-IFN treated CBMC/MT-2 co-cultures suggested that it can reverse the suppression of CBMC proliferation when lymphocytes and monocytes were reconstituted at optimal ratio for antigen-presentation function [8], reducing the requirement for IL-1 [10, 11] in parallel with inhibition of viral transcription [6, 7] and modulation of c-sis/PDGF2 expression [11]. In our *in vitro* model, the first phase of infection should be characterized by allogeneic recognition by both CBMC and PBMC of the virus producing MT-2 line. Therefore effector cells such as CTL (cytotoxic T-lymphocytes) and LAK (IL-2 activated killer cells) should be generated. However, virus infection caused a severe impairment of the T-cell function, as is shown by a decrease of proliferative response of CBMC and PBMC to allogeneic virus donor MT-2 cells and by the lack of natural or specific cytolytic activity of virus-infected effector cells [8, 21–23]. These observations may, in part, explain the aggressive behaviour displayed by this retrovirally induced neoplastic process. Moreover, previous studies have shown that HTLV-I infected T-cells can be the target of cell-mediated cytotoxicity afforded by fresh large granular lymphocytes and LAK cells [24–26]. In addition, IFN treatment can boost the cytotoxic activity of fresh PBMC to virus infected cells in a short term ^{51}Cr release assay [27]. All these data suggest that the antiviral antigen-dependent or natural immunity of PBMC and CBMC are inhibited by the virus itself or by virus-mediated factors released during the co-culture. One of the mechanisms of protection afforded by IFNs could imply a selective effect of IFNs by targeting both mature and immature T-cells in the early phase of infection. Studies performed on T-cell subsets derived from CBMC suggested that β-IFN would protect the preferential target cell of HTLV-I, i.e. CD4 +, rather than differently affecting the individual CD4 + and CD8 + subpopulations or down-modulating the phenotype expression of T-cells. In addition, CD8 + were sensitive to IFN treatment as well as the CD4 + subset, although at different times of culture, and both mirrored, in terms of percentage of inhibition, the protective effect afforded by IFN in the *in toto* co-cultures. The lower percentage of inhibition in CBMC/MT-2 co-cultures could be due to the presence of natural surveillance mechanisms in the whole cell population underlying the interactions between different T-cell subsets [25]. In conclusion, evidence is here provided supporting rational bases for an effective prevention therapy in the early phase of HTLV-I infection, in order to contribute to serum conversion from positive into negative in healthy carriers.

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