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DIFFERENTIAL INTERFERENCE OF ALPHA, BETA OR GAMMA INTERFERONS WITH HTLV-I INTEGRATION AND EXPRESSION *IN VITRO*

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Summary. - *Alpha, beta and gamma interferons (IFNs) can exert a powerful and direct antiviral activity against HTLV-I and can also modulate positively some cell-mediated immune functions of the host cell. These multiple effects of IFNs can induce a long-lasting inhibition of viral infection in recipient cells, probably by "priming" the host cell to an active antiviral competence. It has to be underlined that each IFN was active differentially on the replicative cycle of HTLV-I, thus suggesting the possibility of a complementary action of IFNs in inhibiting HTLV-I infection. This might be relevant to a possible therapeutical approach for prevention of HTLV-I related diseases.*

KEY WORDS: HTLV-I, interferon, retrovirus replication.

Riassunto (Gli interferoni alpha, beta e gamma interferiscono in modo differenziato con l'integrazione e l'espressione *in vitro* di HTLV-I). - *Gli interferoni (IFN) α, β e γ esercitano un potente effetto antivirale diretto nei confronti di HTLV-I e inoltre modulano in modo positivo alcune funzioni immuni cellulo-mediate nella cellula ospite. Questi effetti multipli degli IFN inducono una inibizione a lungo termine della infezione con questo virus, probabilmente attivando la cellula ospite ad uno stato di attiva competenza antivirale. E' di particolare interesse il fatto che i tre IFN agiscano in modo differenziato sulle diverse fasi del ciclo replicativo di HTLV-I, suggerendo una possibile complementarietà tra queste biomolecole nella inibizione dell'infezione con HTLV-I. Ciò potrebbe essere rilevante ai fini di un possibile approccio terapeutico per la prevenzione delle manifestazioni patologiche associate a questo retrovirus.*

PAROLE CHIAVE: HTLV-I, interferone, replicazione di retrovirus.

Introduction

Human T cell leukemia/lymphoma virus type I (HTLV-I) is a human retrovirus associated with adult T-cell leukemia (ATL) [1-3], a malignancy of mature T lymphocytes characterized by a $CD3^+$, $CD4^+$, $CD8^-$ $CD11^-$ surface phenotype [4]. In ATL patients T lymphocytes contain one or few copies of integrated provirus sequences in the host cell genome [2, 5]. After integration of the provirus, virus-infected cells progress from an initial growth crisis to polyclonality and later on to predominantly monoclonal cells [6], which *in vitro* but not *in vivo* usually express high levels of viral RNA [7]. A severe impairment and altered regulation of cell-mediated immunity is associated with HTLV-I infection *in vivo* and *in vitro* [8-10], thus contributing to the persistency of infection, which represents a prerequisite for the development of the leukemic state. This immune depression can contribute to the failure of antiviral therapy to control the endogenous spreading of viral particles in the early, latent phase of disease.

Since α, β and γ interferons (IFNs) are known to possess antiviral properties in a number of experimental models [11], it is conceivable that they would influence also the replicative cycle of HTLV-I. An inverse relationship between IFN-γ production in infected cells and HTLV expression was described [12] as well as a suppressive effect of IFNs on HIV (human immunodeficiency virus) replication, the AIDS-related retrovirus [13, 14]. We have recently shown that α and β IFN can protect mononuclear cells derived from cord blood (CBL) or from peripheral blood of adult donors (PBL) from *in vitro* infection with HTLV-I [15, 16]. The protective effect of IFNs is based on multiple mechanisms of action. Alpha, β and γ IFN could

differentially modulate the cell-mediated immune function of infected CBL, partially reversing the virus-induced immune suppression [17]. In particular, IFN-β can contribute to control the alterations of host cell cycle in the early phase of infection [18], whereas α and γ IFNs can delay the clonal expansion of potentially transformed cells in a later phase of infection [17].

Beside their immunomodulating properties, all three types of human IFNs were also found to affect directly the replicative cycle of HTLV-I in the *in vitro* system herein described since they exerted a differential effect on the infectivity, integration and transcription of HTLV-I. Evidence is here provided showing that IFNs could "prime" CBL to a long lasting antiviral competence by cell-type specific activation. This fact suggests that a defined, although mostly unknown, group of cellular genes might be involved in the IFN-induced activation of host cells against HTLV-I. There is experimental evidence that the expression of some growth regulating factors like IL-1β [18, 19], IL-2 [20], TNF [21], GM-CSF [20] and c-sis/PDGF2 [18] is affected by HTLV-I. In particular, the expression of IL-1β and of c-sis/PDGF2 can be differently modulated in CBL by treatment with IFN-β [18]. These experiments provide a rationale for an experimental scheme of therapy with interferons for prevention of mother to foetus transmission and could also be considered as a model of *in vitro* infection of immature mononuclear cells, that might in part mimic the infection of bone marrow precursors.

Materials and methods

Infection of CBL with HTLV-I and treatment with interferons

Human CBL were infected by co-cultivation with lethally irradiated virus-producing cells (MT-2, a CBL-derived T cell line, [22]) at a 5:1 ratio, as previously described [15]. CBL were routinely maintained in long-term culture by supplementing the medium with 20 IU/ml of recombinant IL-2 (kindly provided by Hoffmann-La Roche, Basel, CH). IFN was added to CBL/MT-2 cocultures at the onset of the culture, at the concentration of 10^2 and 10^3 IU/ml. Alternatively, CBL (effector cells) or MT-2 cells (virus-producing cells) were pretreated with IFN overnight before coculturing, in order to distinguish between the boosting effects of IFN on the antiviral response of CBL and a direct antiviral effect on the stable transformed virus-producing MT-2 line. Recombinant α and γ IFNs were kindly provided by Hoffmann-La Roche and purified natural IFN-β by Sclavo (Siena, Italy).

Infection of CBL was evaluated during long-term culture by indirect immunofluorescence for the p19 viral core protein [23]. Integration of provirus sequences in infected cells was evaluated by dot blot analysis on genomic DNA extracted from CBL by the standard proteinase K method, as previously described [15] or on cells directly spotted on nitrocellulose membranes [24]. Filters were then hybridized under standard conditions with the ^{32}P-labelled Sst1-Sst1 fragment [15], derived from pMT-2 plasmid (kindly given by R.C. Gallo, NCI, NIH, Bethesda). This 8.5 kb fragment accounts for almost the entire viral sequences. Autoradiography was performed by using X AR Kodak films.

Transcription of HTLV-I in IFN-treated CBL

The amounts of viral transcripts in cocultured CBL or in the virus-immortalized MT-2 cells were evaluated by Northern blot or dot blot analysis either with total RNA extracted from intact cells by the guanidine-thiocyanate method [25] either with cells directly spotted on nitrocellulose filters [26]. Filters were hybridized under standard conditions as described for DNA blots [15]. Autoradigraphy was performed by using X AR Kodak films.

In a separate experiment a Northern blot was performed on total RNA extracted from isolated adherent (mostly monocytes) and non-adherent (mostly lymphocytes) CBL cocultured with irradiated MT-2 cells and tested 5 days post infection (p.i.). Data were compared with the percentage of infected cells in the culture as evaluated by indirect immunofluorescence for the p19 virus protein.

Results

Inhibition of HTLV-I infectivity in IFN-treated MT-2 cells

Alpha, β and γ IFNs all inhibited *in vitro* the infection of CBL with HTLV-I by affecting different steps of virus replication. The infectivity of MT-2 virus-donor cells was greatly impaired by overnight treatment with 10^3 IU/ml of IFN-α and to a minor extent by IFN-γ (10^2 and 10^3 IU/ml), as shown by a significant reduction of the percentage of CBL positive for the p19 virus core protein by indirect immunofluorescence following infection with IFN-treated MT-2 cells (Table 1). In contrast, overnight pretreatment of MT-2 cells with 10^3 IU/ml of IFN-β did not modify the subsequent infection of CBL. To explore whether the reduced virus transmission was due to inhibition of virus transcription, dot blots were performed on total RNA extracted from MT-2 cells treated with IFNs every 48 h, from day 0 to 6. After overnight treatment with IFNs, the amount of viral transcript was not significantly reduced in MT-2 cells treated with IFN-α, whereas it was slightly reduced after treatment with IFN-β and IFN-γ (data not shown). After six days of treatment, both α and β IFN clearly reduced the amount of viral mRNA when used at the concentration of 10^3 IU/ml (Fig. 1), whereas IFN-γ was effective earlier and at a lower concentration (10^2 IU/ml) (data not shown). A general decrease of fluorescence intensity for the p19 viral protein was also observed in MT-2 cells, although nearly 100% of the cells remained positive, suggesting that the majority of MT-2 cells were

responsive to the antiviral effects of IFNs. However, depression of HTLV-I transcription cannot entirely explain the inhibition of virus transmission, since pretreatment of MT-2 cells with IFN-β did not impair infection of CBL (Table 1), although it reduced the amount of viral mRNA in treated MT-2 cells.

Table 1. - *Inhibition of HTLV-I transmission following overnight pretreatment of immortalized, chronically infected MT-2 cells with α, β or γ interferon, evaluated as reduction of infection in different cultures of recipient CBL, tested 1 week post infection by indirect immunofluorescence for the p19 viral core protein*

p19⁺ CBL 1 week p.i. (%)			
Controls	IFN pretreated MT-2 cells	IFN type	Δ (%)
14.4	9.0	α 10^3 IU/ml	- 5.4 (*)
7.6	4.7	β 10^3 IU/ml	- 2.9
26.9	13.2	γ 10^2 IU/ml	- 13.7 (*)

(*) Significance was calculated by χ^2 analysis

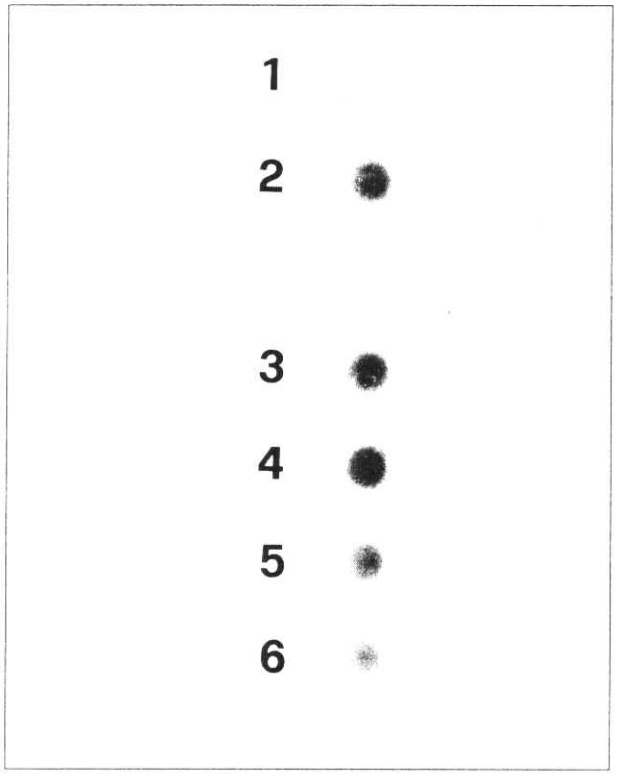

Fig. 1. - Effect of α and β interferon on transcription of HTLV-I mRNA in the immortalized chronically infected MT-2 cells. Cells were treated every 48 h for 1 week with recombinant α2 interferon (10^2 IU/ml, line 3; 10^3 IU/ml, line 4) or natural purified β interferon (10^2 IU/ml, line 5; 10^3 IU/ml, line 6). Negative (K562 cells) and positive (untreated MT-2 cells) controls are shown in the line 1 and 2, respectively. 3 μg of total RNA were spotted on nitrocellulose fitter for each sample.

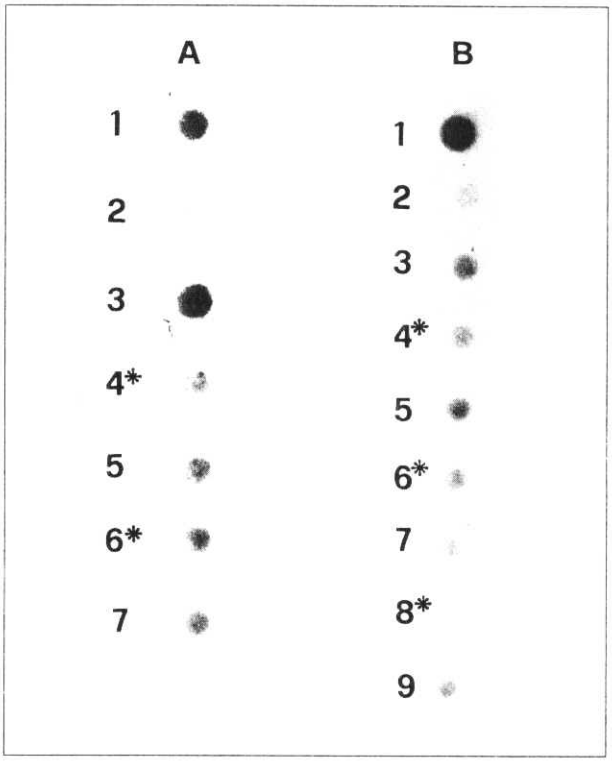

Fig. 2. - Presence of integrated provirus sequences in CBL cocoltured with irradiated MT-2 cells and tested 2 weeks post infection. CBL were preatred (*) overnight before the onset of the cocolture or treated at day 0 with 10^3 IU/ml of recombinant α2 (lines 4-5A) or natural purified IFN-β (lines 6-7A), or recombinant IFN-γ (10 IU/ml, lines 4-5B; 10^2 IU/ml, lines 6-7B; 10^3 IU/ml, lines 8-9B). Positive (MT-2 cells, lines 1) and negative (non infected CBL, lines 2) controls are inserted in the figure. Control CBL/MT-2 cultures are shown in line 3A and 3B, respectively. Genomic DNA was extracted from the cells 2 weeks post infection and 3 μg were spotted on nitrocellulose filters for each sample.

Cell-type specific antiviral effects of interferons

The antiviral effects of IFNs appeared also to be mediated by a cell-type specific induction of an antiviral competence. In fact, ovenight pretreatment of CBL with all three types of IFNs before coculturing with MT-2 cells resulted in a similar inhibition of CBL infection as when adding IFN as a single treatment at the onset of the coculture. Moreover, the protective effect of IFNs was remarkably evident until 4 weeks post infection (p.i.) [15-17] and was not significantly improved by weekly repeated treatments. In IFN-boosted CBL cultures, the amount of integrated provirus was reduced when compared with untreated controls, starting a few hours p.i. and being still remarkable 2 weeks p.i. (Fig. 2). However, within 24 h p.i., a clear cut reduction of viral mRNA was found only with IFN-β (data not shown). Two weeks later, while p19 positivity remained significantly low in IFN-treated CBL, especially after treatment with α or β IFN, the amount of viral transcripts in CBL treated with 10^3 IU/ml of α or β IFN remained constant or was slightly increased, depending on different CBL donors (Fig. 3). This suggests that viral mRNA might accumulate in the cell because of ineffective

viral protein synthesis. In contrast, late viral transcription was inhibited when 10^2 IU/ml of IFN-γ were used, but not with 10^3 IU/ml. Data showing the inhibitory effects of IFNs on CBL/MT-2 cocultures are summarized in the Table 2.

Role of monocytes in the early phase of infection of CBL with HTLV-I

The increment of the relative percentage of monocytes (CD14 phenotype) is a constant event in the early days p.i. [11]. Northern blot analysis on isolated monocyte (Mo) or lymphocyte (Ly)-enriched subpopulations of CBL revealed that the presence of HTLV-I transcripts in Mo, 5 days p.i., is approximately 10-fold higher than in lymphocytes, being the number of virus positive cells in the Mo subset only doubled in comparison with Ly (Fig. 4). Previous experiments showed that treatment of CBL with IFNs at time 0 maintained the relative percentage of the Mo subset to levels comparable with those of non infected control cultures [17]. Preliminary experiments performed on Percoll-fractionated CBL [27] showed that null and T cell fractions were infected by HTLV-I at 3-fold higher proportion that monocyte fractions, whereas the LGL-enriched fraction was resistent to the infection, according with previous results [28]. However, the percentage of total infected cells in Percoll-isolated fractions was comparable with that of infected cells in the whole CBL population (10%). Alpha and β IFN were differentially active on CBL subsets when compared with the overall inhibition of HTLV-I infection mediated by IFNs in the whole CBL cultures. In fact, 1 week p.i. both IFNs induced a 6 to 20-fold increase of the relative number of virus$^+$ cells in the T lymphocyte fraction, whereas IFN-β (but not IFN-α) increased this percentage also in the LGL-enriched fraction (CD16 phenotype) and to a minor extent in the Mo (CD14 phenotype) subset. This increment of the number of virus$^+$ cells was prevented when Mo were added as accessory cells to autologous LGL or T-cells (data not shown).

Table 2. - *Influence of α, β and γ IFN on HTLV-I transmission, integration and expression in CBL/MT-2 cocoltures after one single treatment on day 0*

IFN	Virus *(a)* transmission	Provirus *(b)* integration	Transcription early *(c)*	Transcription late *(d)*	p19 *(e)* (%)
a	↓↓	↓↓↓	↓	↑	↓↓
β	no change	↓↓↓	↓↓↓	↑	↓↓↓
γ	↓↓	↓↓	↓↓	↓	↓

(a) Evaluated in terms of infectivity of IFN pretreated MT-2 cells; *(b)* Dot blots on genomic DNA from infected CBL, 2 weeks p.i.; *(c)* Northern blots, 6-18 h p.i.; *(d)* Dot blots, 2 weeks p.i.; *(e)* Indirect immunofluorescence, 2-4 weeks p.i.

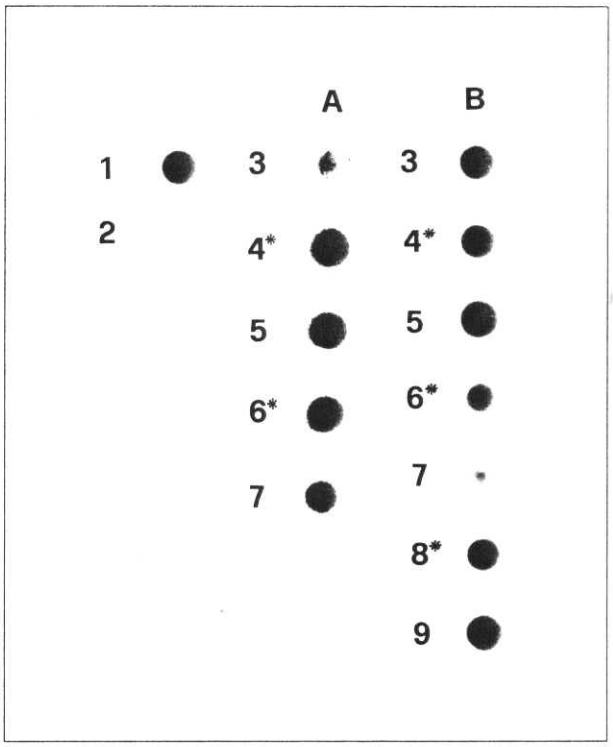

Fig. 3. - Transcription of HTLV-I mRNA in CBL cocultured with irradiated MT-2 cells and tested 2 weeks post infection. CBL were pretreated (*) overnight before the onset of the cocolture or treated at day 0 with recombinant α2 (10^3 IU/ml, lines 4-5A), natural purified IFN-β (10^3 IU/ml, lines 6-7A), or IFN-γ (10 IU/ml, lines 4-5B; 10^2 IU/ml, lines 6-7B; 10^3 IU/ml, lines 8-9B). Positive (MT-2 cells, line 1) and negative (non infected CBL, line 2) controls are inserted in the figure. Total RNA was extracted from the cells 2 weeks post infection and 3 μg were spotted on nitrocellulose filters for each sample.

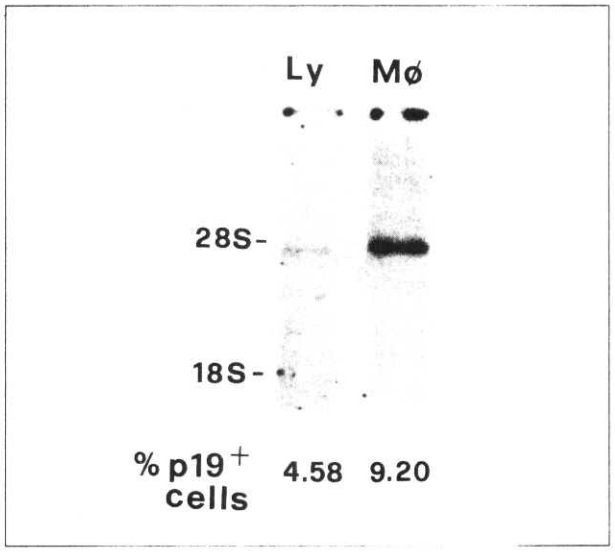

Fig. 4. - Different permissivity of monocyte (Mø) and lymphocyte (Ly)-enriched sub-populations of CBL to early infection with HTLV-I. The abundance of viral transcripts in the Northern blot is apparently associated with a few number of infected cells in the Mo subset, as evaluated by indirect immunofluorescence for the p19 virus core protein. Total RNA was extracted from the cells 5 days post infection and 15 μg RNA were run for each sample.

Discussion

The overall protection against HTLV-I infection induced *in vitro* by treatment with α, β and γ IFN apparently resulted from inhibition of different steps of virus replication in the host cell. The most clearcut inhibition was observed after treatment with α or β IFNs. However, IFN-α effectively prevented virus transmission, whereas IFN-β did not. Nevertheless, all three types of IFN induced a persistent antiviral state in CBL exposed to HTLV-I. IFN-γ was effective at a lower concentration than α or β IFN and was also the most powerful up-regulator of the cell-mediated immune function of CBL, which was otherwise profoundly depressed after HTLV-I infection [17]. However, the direct antiviral effect of IFN-γ was less impressive as compared to that of α or β IFN and was mostly limited to early days p.i. In contrast, the antiviral state induced in CBL by a single treatment with 10^3 IU/ml of either α or β-IFN resulted in a 50 to 80% reduction of the percent number of infected cells until 4 weeks p.i. [15, 17], with a parallel decrease of the amount of integrated provirus in the coculture [15, 16] (Fig. 2). While IFN-α significantly contributed to reduce HTLV-I transmission to CBL from treated MT-2 cells, IFN-β mostly impaired virus integration and transcription from integrated provirus during early phase of infection, and presumably inhibited also the synthesis of viral proteins in a later phase, as shown by accumulation of viral mRNA in the absence of any increase of the percent number of virus$^+$ CBL. Experiments are in progress to verify whether IFN-β directly affects the transcription rate of viral mRNA or its stability in different phases of infection. The fact that multiple treatments with IFNs are required for MT-2 cells to achieve similar degree of inhibition of viral transcription as that observed in whole CBL cultures (but not in isolated CD4$^+$ or CD8$^+$ T cells, unpublished results) after one single treatment, suggests the possibility that in primary mononuclear cells a cooperation among T cells and monocytes would be required to enhance the IFN signal pathway . Macrophages, that might represent a reservoir for HTLV-I, like for HIV [29, 30], are involved early in the antiviral response of CBL, since a subset among cells expressing the the CD14 phenotype was found to be intensively positive for HTLV-I and since addition of autologous Mo to lymphocytes partially protected these cells from infection. Moreover, IFN-β, unlike IFN-α, was found to modulate virus uptake in the LGL and Mo compartment. Whether this uptake is a consequence of enhacement of the killing activity of these cells remains to be defined.

Data herein described provide an experimental support to recent reports of positive therapeutical results obtained after treatment of ATL patients with β [31, 32] and γ IFN [31]. Moreover, a deeper insight into the cellular and molecular mechanisms underlying the antiviral effects of IFNs against HTLV-I infection would be a prerequisite for optimizing combined antiviral and immunomodulating therapy for prevention of retrovirus-induced diseases in seropositive individuals [33].

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