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Suppressive effects of interferons on the production and release of human T-lymphotropic virus type-I (HTLV-I)

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Summary. The effects of human α-, β-, or γ-interferon (IFN) on the replication and production of human T-lymphotrophic virus type-I (HTLV-I) were investigated in a human T-cell line, MT-2. Virus transmission and production estimated by syncytium formation and HTLV-I-associated reverse transcriptase (RT) activity were strongly suppressed in the presence of α- and β-IFN, but not γ-IFN. However, the expression of virus specific proteins gp46 but not p19, p24, p28, p36, and gp68 was affected with IFNs as revealed by Western blotting analysis. Electron microscopic observations showed that some of the HTLV-I particles were trapped in the intracellular vacuoles in the presence of high doses of α- or β-IFN. Continuous supply of IFNs appeared to be essential for the constant suppression of RT activity. These results suggest that α- and β-IFN do not inhibit HTLV-I gene expression strikingly but suppress processing or assembly of virus proteins and/or releasing of virions in the late phase of maturation.

Introduction

Interferons (IFNs) comprise a family of gene products which were first identified by their abilities to prevent the virus infections [1]. The gene structures of IFNs and the amino acid sequences of the encoded proteins have been characterized in detail [2, 5]. IFNs are classified into three types, alpha, beta, and gamma, according to their antigenicities. In human there are 20 species of α-IFN but only one of β-IFN and of γ-IFN [5]. IFNs have been reported to have various biological activities including not only antiviral actions but also inhibition of cell growth and proliferation[3], regulation of the expression of specific genes [4], modulation of cell differentiation and activation of various types in the immune systems [5].

Human T-lymphotropic virus type-I (HTLV-I), which is a type C retrovirus isolated from adult T-cell leukemia/lymphoma (ATLL), can immortalize T lymphocytes and fuse the fibroblasts or epithelial cells to produce large syn-

cytium in vitro [6]. The same virus has been related to certain chronic myelopathies in the tropics and Japan, called HTLV-I-associated tropical spastic paraparesis (HTLV-I/TSP) or HTLV-I-associated myelopathy (HAM) [7]. Several immunological abnormalities have been observed in ATLL or HAM patients, such as alterations of peripheral blood lymphocyte (PBL) subpopulations, IL-2 independent proliferation of T-lymphocytes or autologous proliferative response (APR) in vitro [6], and indiscriminant helper functions [8]. Furthermore, the expression of HTLV-I-related viral proteins as well as virus production is tightly restricted in fresh lymphocytes of ATLL patients and virus carriers in vivo [9]. This suggests that the expression of viral-specific proteins or the virus production are negatively feedbacked in response to cytokines or lymphokines to evade from immunological surveillance in vivo. Therefore, it is reasonable to hypothesize that immunological surveillance mechanisms play an important role to control HTLV-I infection and transmission in the early stage and progression into leukemic stage. The purpose of the present study is to examine the role of human α-, β-, and γ-IFN on the replication, production, and transmission of HTLV-I in vitro.

Materials and methods

Cell culture

MT-2 [10], a human cord T cell line established by co-culture with fresh ATL cells; Rab-3 [11], a rabbit-lymphoid cell line, established by co-culture with lethally irradiated MT-2 cells, and TALL-1 [12], a human leukemic T cell line, were cultured in RPMI-1640 medium supplemented with 15% heat-inactivated fetal calf serum (FCS).

The monolayer culture line used as an indicator of syncytium formation was S^+L^- CCC [13], a clonal cell line derived from feline kidney fibroblasts transformed by Moloney murine sarcoma virus (Mo-MSV) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. All these cells were cultured in a humidified 7.0% CO_2 atmosphere at 37 °C.

IFNs used were recombinant type human α-IFN (rIFN) 2a (Roferon-A, specific activity: 2×10^8 I.U./mg protein) kindly provided from Japan-Roche Co. Ltd., affinity purified natural type human β-IFN (nIFN) (BM532, specific activity: more than 2×10^7 u/mg protein), and recombinant type human γ-IFN (rIFN) (GI-3, specific activity: more than 1×10^7 u/mg protein) from Toray Co. Ltd. Japan, and natural type human α-IFN (n-IFN) (Sumiferon, specific activity: 1 to 2×10^8 u/mg protein) from Sumitomo-Pharmaceutical Co. Ltd. Japan.

In order to estimate the suppressive effects of each IFN treatment on the growth of MT-2 cells, 2×10^5 cells/ml were cultured for 48 or 72 h in the presence of various doses of IFNs and viable cells were counted by trypan blue exclusion.

Syncytia formation assay

5×10^4 S^+L^- CCC cells/well were cultured overnight in 24-well tissue culture plate, followed by polybrene treatment (2 μg/ml, Sigma) for 30 min. Then, $5–50 \times 10^4$ MT-2 cells along with different doses of each IFN were inoculated in each well. After 3-day culture, cells were fixed with methanol and stained with Giemsa. Numbers of syncytia containing more than ten nuclei were counted under an inverted microscope. S^+L^- CCC cells rarely formed syncytium spontaneously and usually possessed less than five nuclei [6].

Immunofluorescence assay

MT-2 cells treated with various units of IFNs were reacted with anti-HTLV-I positive human sera and FITC-conjugated goat anti-human IgG and analyzed by an immunofluorescence microscope. Titers of each serum were determined with non-treated MT-2 cells by the same method.

Electron microscopy

For electron microscopic observation, IFN-treated cells were processed as reported previously [14]. Briefly, pelleted cells were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol, and embedded in an epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined under a Hitachi H-300 type electron microscope.

Western blotting analysis

Western blotting analysis was performed according to the technique of Towbin [15]. Following 12.5% polyacrylamide gel electrophoresis of 40 μg of cellular protein lysate from each culture, banded proteins were electrophoretically transferred to nitrocellulose membranes and treated with an anti-HTLV-I-positive rabbit serum (1:1280). Immunoreactive bands were visualized after the incubation with biotinylated donkey anti-rabbit Ig, and streptavidin-biotinylated alkaline phosphatase, following the reaction with the substrate of nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in diethanolamine buffer (Amersham Japan Co. Ltd.).

Reverse transcriptase assay

The released virus particles were estimated by the detection of reverse transcriptase (RNA-dependent DNA polymerase) activity in the culture medium. Twelve ml of culture medium harvested at a concentration of 1×10^6 cells/ml were centrifuged at 8,000 rpm for 10 min (RPRW20–3, Hitachi) and then the supernatant was also centrifuged at 36,000 rpm for 3 h (Type 50Ti, Beckmann) at 4 °C. The pellet obtained was resuspended in a buffer containing 50 mM Tris-HCl (pH 7.8). The reverse transcriptase activity in the suspension was assayed in a standard reaction mixture according to Rho et al. [16].

To clarify the recovery effect after removal of IFNs, 5×10^5 MT-2 cells/ml were plated in medium containing 25,000 u/ml of α-rIFN 2a. Three days later, IFN was removed and at every 12 h, medium was collected and virus production was analyzed as mentioned above together with non-treated cells as control.

Results

Syncytium forming activity of HTLV-I in the presence of IFNs

As shown in Figs. 1 and 2, α-rIFN 2a, α-nIFN, and β-nIFN strongly inhibited the syncytia formation of S^+L^- CCC cells in a dose-dependent manner. Especially, syncytia formation was suppressed nearly to the background level in cultures containing 25,000 u/ml of α-rIFN 2a or α-nIFN. On the other hand, weak inhibition was observed by γ-rIFN.

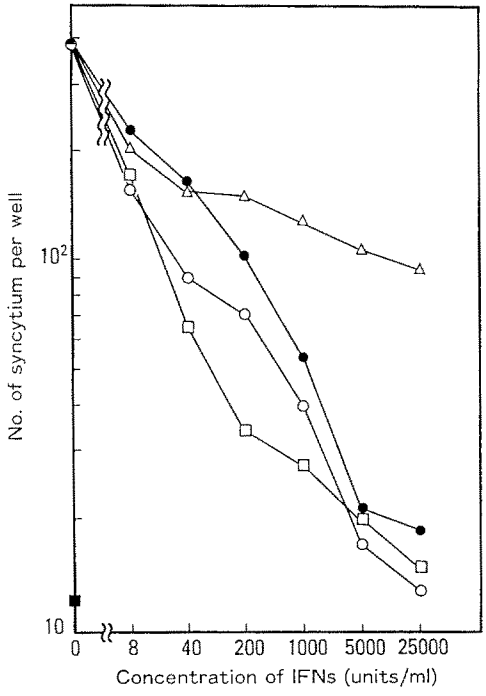

Fig. 1. Effect of IFNs on syncytium formation of S^+L^- CCC cells co-cultured with MT-2 cells for 3 days. □ α-nIFN, ○ α-rIFN 2a, ● β-nIFN, △ γ-rIFN, ■ culture medium only, ◓ MT-2 non-treated

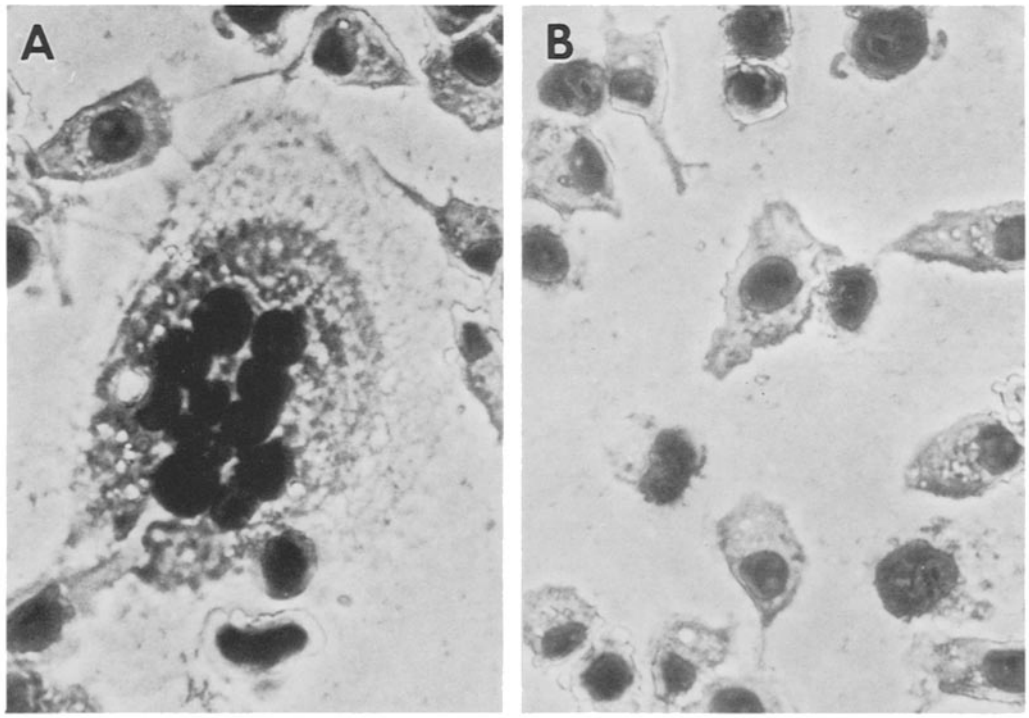

Fig. 2. S^+L^- CCC cells co-cultured with MT-2 cells in the absence **(A)** or in the presence of 5,000 u/ml of α-rIFN 2a **(B)** for 3 days. × 500

Effects of IFNs on the growth of MT-2 cells

Growth rates of MT-2 cells were slightly suppressed in the presence of α-rIFN 2a, α-nIFN, β-nIFN, or γ-rIFN after 72 h of culture (Fig. 3). Number of viable cells in cultures containing 25,000 u/ml of each IFN, in which growth suppressive effect was the maximum, ranged from 45 to 65% as compared with the control value. Growth inhibitory effects of each IFN on MT-2 cells were dose-dependent.

Expression of HTLV-I-related viral proteins in the presence of IFNs

Almost 100% of MT-2 cells from 3 day cultures, containing 8, 40, 200, 1,000, 5,000, 25,000 u/ml of α-rIFN 2a, α-nIFN, β-nIFN, or γ-rIFN, reacted with ATL patients' sera as strongly as control cultures by indirect immunofluorescence (data not shown).

In Western blotting analysis, protein bands of HTLV-I such as p19, p24, p28, p36, gp46, and gp68 were clearly detected in each IFN- or non-treated MT-2 cells, but not TALL-1 cells (Fig. 4). Intensities of each protein band were hardly affected in the 3-days cultures containing IFNs. However, a slight dose-dependent decrease of the gp46 density was detected after treatment with each IFN.

HTLV-I virion production and release in the presence of IFNs

Ultrastructurally, the general features of virus particles as well as their distribution and numbers were not strikingly affected in MT-2 cells cultured for 3 days in the presence of 5,000 u/ml of α-rIFN 2a, β-nIFN, or γ-rIFN when compared with control cultures, although intracellular vacuoles containing virus

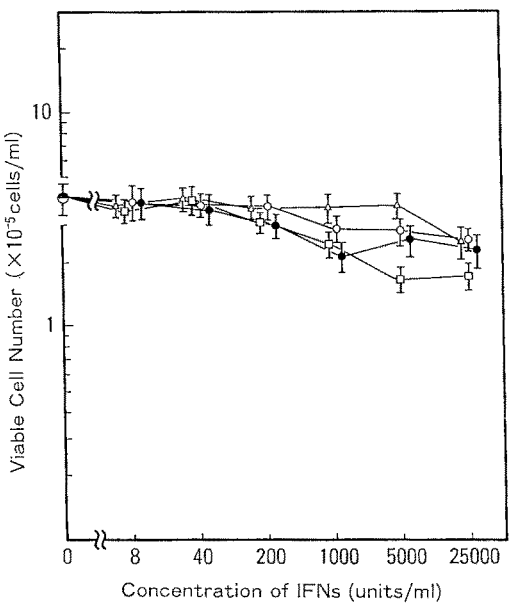

Fig. 3. Effect of IFNs on the growth of MT-2 cells. Viable cell counts unstained with tripan blue at 72 h culture after inoculation of 2.0×10^5 cells/ml of MT-2 cells. Bars indicate standard errors. □ α-nIFN, ○ α-rIFN 2a, ● β-nIFN, △ γ-rIFN, ◓ MT-2 non-treated

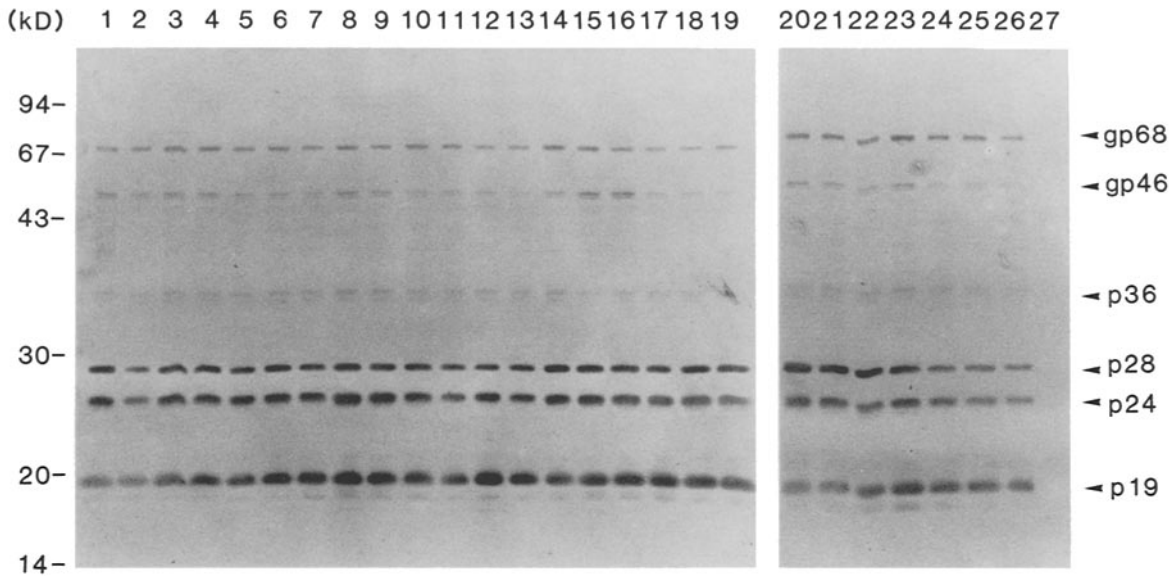

Fig. 4. Effect of IFNs on the HTLV-I-related viral specific protein synthesis. Western blotting analysis of viral proteins and glycoproteins of MT-2 cells cultured for 3 days in the presence or absence of IFNs. *1* MT-2 cells as positive control; *2–7* (α-rIFN 2a), *8–13* (β-nIFN), *14–19* (γ-nIFN) *20–26* (α-nIFN); MT-2 cells treated with 8, 40, 200, 1,000, 5,000, or 25,000 u/ml of each IFN, in this order. *27* TALL-1 cells as negative control

particles were occasionally observed. The degenerative changes were prominent in the presence of 25,000 u/ml of IFNs. The total number of cell-associated extracellular virus particles was not evidently changed, but interestingly, "trapping" of virus particles in the intracellular vacuoles was frequently observed with 25,000 u/ml of α-rIFN 2a (Fig. 5) or β-nIFN.

Reverse transcriptase (RT) activity was decreased dose-dependently in MT-2 cells cultured for 3 days in the presence of α-rIFN 2a, α-nIFN, or β-nIFN and was about one-tenth the control values with 5,000 u/ml of α-rIFN or α-nIFN (Fig. 6). α- and β-IFN were more inhibitory to RT activity than γ-rIFN.

The suppressive effect on the virus production disappeared rapidly, when IFN was removed from culture medium of MT-2 cells. The production of HTLV-I had recovered to normal levels within 72 h after removal of 25,000 u/ml of α-rIFN 2a as estimated by RT activity. On the other hand, there was no effect on the production of HTLV-I in rabbit lymphoid cells, Rab-3 (Fig. 7).

Discussion

The present results show that HTLV-I transmission determined by syncytium formation in S^+L^- CCC cells was strikingly suppressed with α-IFN and β-IFN. This suppression was not limited to only a single α-IFN subtype, but also α-nIFN containing various subtypes. In the recent study of D'Onofrio et al. [17], β-IFN reduced HTLV-I transmission and integration in human cord blood

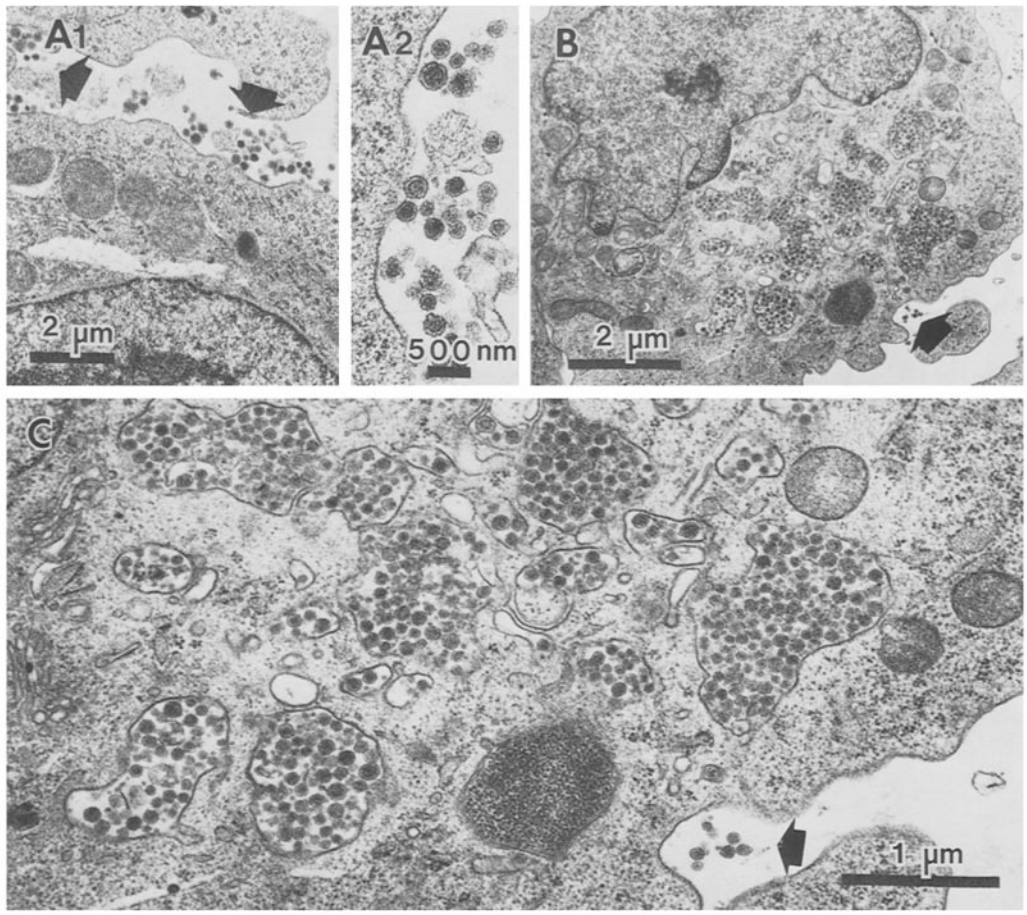

Fig. 5. Effect of α-rIFN 2a on the production and distribution of virus particles in MT-2 cells **B, C** cultured for 3 days in the presence of 25,000 u/ml of α-rIFN 2a, **A1, A2** cultured in the normal control medium for 3 days. Note extracellular virus particles (arrows). Uranyl acetate and lead citrate stains

lymphocyte cultures. As for these suppressions, several possible spots may be considered: such as gene expression of HTLV-I genome including transcription and translation, assembly or release of viral particles, or fusion steps involved in syncytium formation. The present observation may be related to the phenomenon described by Billiam et al. [18, 19] and Friedman et al. [20], in murine leukemia virus (MuLV)-infected mouse fibroblast cells. Then, human IFNs did not block HTLV-I specific protein synthesis except for gp46, which was weakly suppressed dose dependently, but markedly inhibited virus production as determined by RT assay. These results suggest a direct effect of IFNs on virus maturation or release from the cell surface. Inhibitory effect on the production of gp46, which is a glycoprotein processed from gp68, localized in the viral envelope, suggests a possibility that influences on endogenous or viral proteases lead to impairment of virus maturation such as protein processing, assembly,

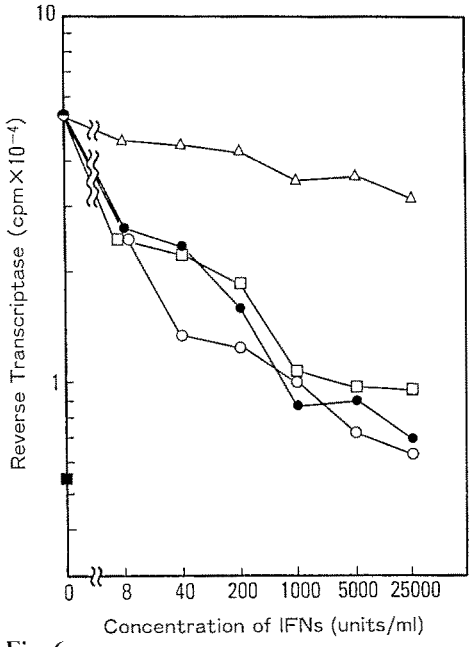

Fig. 6

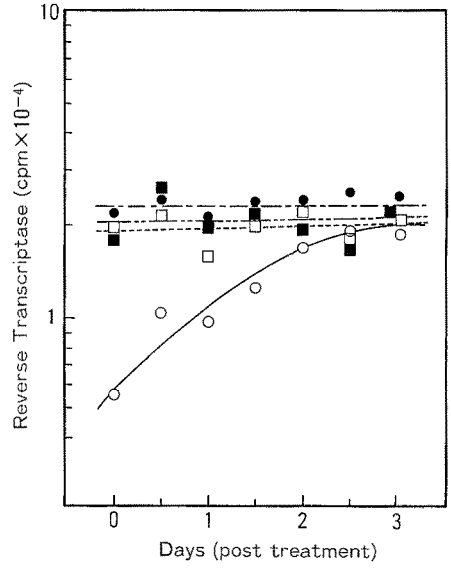

Fig. 7

Fig. 6. Reverse transcriptase activities of HTLV-I in the culture medium of MT-2 cells cultured for 3 days in the presence or absence of IFNs. ○ α-rIFN 2a, □ α-nIFN, ● β-nIFN, △ γ-rIFN, ■ culture medium only, ◓ MT-2 non treated

Fig. 7. Effect of α-rIFN 2 a on virus production after removal of IFN from MT-2 or Rab-3 culture. After 3 days culture in the presence of 25,000 u/ml of α-rIFN 2a, IFN was removed and cells were cultured with IFN-free medium. At the time indicated (days post removal of α-rIFN 2a), the mediums of MT-2 cells (○) or Rab-3 cells (□) were collected and analyzed with RT assay. ● MT-2 non-treated, ■ Rab-3 non-treated

or virus release. Electron microscopic observation seemed to support this hypothesis, revealing "trapping" of HTLV-I virion particles in the cytoplasmic vacuoles. This may be analogous to murine leukemia viruses trapped at the outer cell surface with IFN treatment [19].

Although the amount of released extracellular HTLV-I virus is depressed, the number of cell-associated type-C virus, as estimated by electron microscopic observation, was little affected if at all [21]. The similar results were obtained with another virus-cell system which was verified with radiolabeled type-C murine oncorna-viruses (MuLV, MSV) in the cell homogenate and IFN treatment most likely inhibited virus release [18, 19]. It is also possible in the case of HTLV-I that IFN-induced antiviral proteins such as 2′,5′A synthetase, protein kinase of phosphodiesterase might interact directly or indirectly with viral glycoproteins or cellular membrane proteins to inhibit virion release and interfere HTLV-I transmission as reported [22, 23].

Recently, a case of adult T-cell leukemia was reported, in which α-IFN

therapy was highly effective and circulating α-IFN as measured by a radioimmunoassay was significantly lower than in age- and sex-matched healthy controls [24]. Examination of consecutive sera from a patient of acute encephalopathy associated with the presence of human immunodeficiency virus type-I (HIV-I) antigen and seroconversion to anti-HIV-I antibody, revealed that circulating α-IFN became detectable with the appearance of HIV-I antigen, and was not detectable after neurological symptoms resolved [25]. These suggest that low levels of circulating α-IFN in the patient may be important from both pathogenetic and therapeutic standpoints. However, in such a low dose of circulating α-IFN, direct effect of IFN on the growth of virus infected cells would not be expected from the present data. It is now well established that α-IFN or β-IFN can up-regulate class-I major histocompatibility (MHC) antigens, but not class-II, while γ-IFN can up-regulate not only class-I but class-II also. These effects should augment the T-cell recognition of foreign antigen and the associated T-cell functions. Thus, T-cell cytotoxicities, both allogeneic and virus specific, will be enhanced, when cells are treated with IFNs [4].

On the other hand, human γ-rIFN exerted only a little effect on HTLV-I production in vitro as determined by RT activity, if any, since the apparent slight suppression of RT activity is comparable to the growth inhibition (Fig. 3). However, suppression of syncytium formation by HTLV-I is not small enough to counterbalance with the growth inhibition. This suggests that physiological changes induced by γ-IFN in the target S^+L^- CCC cells of syncytium formation assay form anti-viral state and reduce HTLV-I transmission.

It is reported recently that expression of core protein of HTLV-I p19 in T lymphocytes transformed by co-culture with lethally irradiated HUT102 or MT-2 cells is inversely proportional to constitutive γ-IFN production [26]. In the present study, the expression of core proteins of HTLV-I p19, p24, and p28 was not affected by γ-IFN and there was no relationship between HTLV-I expression and γ-IFN doses except for gp46. One explanation of this discrepancy may be due to γ-IFN resistance of MT-2 cells. However, it is also possible that γ-IFN did not directly suppress the HTLV-I expression but changes of cell population in vitro was induced with the selection of a fast growing HTLV-I producer cells. On the other hand, another population of normal γ-IFN producing cells, which reacted against HTLV-I or its producer cells, were selected out or dead eventually. It may cause such phenomena as the inverse relationship between HTLV-I expression and γ-IFN production.

Finally, α- and β-IFN are strikingly suppressive against HTLV-I production and transmission. However, the reversibility of the inhibition upon IFN removal will require continuous IFN therapy in clinical application, which may be accompanied by toxicity and negative effects on patients' immune system. Gene or protein engineering technology would overcome these difficulties with the modification or reconstruction so as to concentrate or stabilize the effect on the target cells in the future.

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