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RECOMBINANT HUMAN INTERFERON GAMMA SUPPRESSES HTLV-III REPLICATION *IN VITRO*

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Effect of human interferon gamma (rINFγ) on HTLV-III replication was evaluated quantitatively via a novel infection system using HTLV-I-carrying MT-4 cells. Treatment of HTLV-III-infected MT-4 cells with different concentrations (1-1,000 U/ml) of rINFγ, which did not affect the growth or viability of uninfected cells, significantly blocked the appearance of immunofluorescent antigens of HTLV-III and the virus-induced cytopathic effect in a dose-dependent manner. A plaque assay was applied to measure the exact amount of viral particles released from HTLV-III-infected MT-4 cultures either untreated or treated with rINFγ after infection. The number of plaques per dish decreased with increasing drug concentrations. About 50% and 80% of HTLV-III replication were inhibited by the addition of 100 and 1,000 U/ml of rINFγ, respectively. The effects of INF were observed by day 5 of incubation with the chemical. However, longer treatment of cells with rINFγ permitted a gradual increase in viral replication. Re-addition of fresh INF into cultures did not change this pattern significantly.

The acquired immune deficiency syndrome (AIDS) is a newly described disorder characterized by extensive immune depression that predisposes patients to life-threatening opportunistic infections and malignant neoplasms (Gottlieb *et al.*, 1981; Siegal *et al.*, 1981). Human retroviruses such as lymphadenopathy-associated virus (LAV) (Barré-Sinoussi *et al.*, 1983), human T-lymphotropic virus type III (HTLV-III) (Popovic *et al.*, 1984*b*) and AIDS-associated retrovirus (ARV) (Levy *et al.*, 1984) have been isolated from patients with AIDS or AIDS-related complex (ARC). These viruses are now believed to be variants of the same virus (Klatzmann *et al.*, 1984*a*; Ratner *et al.*, 1985) and destroy $OKT4^+$ (helper/inducer) T-lymphocytes (Klatzmann *et al.*, 1984*b*; Popovic *et al.*, 1984*a*).

Recent studies suggest that substances inhibitory for reverse transcriptase, such as suramin (Mitsuya *et al.*, 1984), antimoniotungstate (HPA-23) (Dormont *et al.*, 1985) and phosphonoformic acid (Sandström *et al.*, 1985) have suppressive effects on the replication of HTLV-III/LAV *in vitro*. Moreover, some other compounds inhibit HTLV-III/LAV replication, *e.g.*, rivavirin (McCormick *et al.*, 1984) and 3'azido-3'deoxythymidine (Mitsuya *et al.*, 1985). These compounds appeared to block viral infection at steps other than that of viral reverse transcriptase. Since interferon is known to suppress replication of many animal retroviruses either *in vitro* or *in vivo* (Pitha *et al.*, 1982; Aboud and Hassan, 1983; Sen *et al.*, 1984; Gresser *et al.*, 1969), it might also inhibit HTLV-III/LAV infections that are prevalent in AIDS patients. Indeed, Ho *et al.* (1985) have reported that recombinant human interferon (rINF) alfa-A suppresses HTLV-III replication *in vitro*, using normal peripheral blood mononuclear cells as responder cells to HTLV-III/LAV infection. Previously, we reported that HTLV-I-carrying cell lines such as MT-4 were markedly permissive for HTLV-III/LAV infection and further showed a strong cytopathic effect (CPE) (Harada *et al.*, 1985*a*). Based on these unique characters of MT-4 cells in terms of response to HTLV-III/LAV infection, we have developed a quantitative assay for HTLV-III/LAV using a plaque-forming assay and a proliferation assay with ^{3}H-thymidine (Harada *et al.*, 1985*b*). In this report, we attempt to investigate the suppressive effect of rINF gamma (rINFγ) on HTLV-III infection, using our quantitative assay systems.

MATERIAL AND METHODS

Virus and cells

Molt-4/HTLV-III cells were adjusted to 3×10^5 /ml in RPMI 1640 medium supplemented with 10% decomplemented fetal calf serum (FCS), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (complete medium), and cultured for 4 days at 37°C.

After the cells had been spun down at 1,500*g* for 10 min, supernatant was filtered through a 0.22-μm Millipore membrane. A small volume of this viral preparation (1 ml) was stored at −80°C until use. The titer of this viral preparation was $10^{4.2}$ 50% tissue culture infectious dose ($TCID_{50}$) per ml assayed by infecting MT-4 cells. The HTLV-I-carrying cell line MT-4 was maintained at 37°C in complete medium. Viable cells, which were counted by the Trypan blue dye exclusion method, were adjusted by sub-culturing to 3×10^5 / ml twice a week.

Chemical

Recombinant human gamma interferon (rINFγ) was provided by Kyowa Hakko, Tokyo, Japan, in a frozen-dry condition. The chemical was dissolved immediately before the experiments and diluted with complete medium.

Procedure for treatment of HTLV-III-infected MT-4 cells with rINFγ

MT-4 cells were suspended at a concentration of 6×10^5/ml, 1 ml of cell suspension was placed into a 24-well culture plate and similar volumes of 0, 2, 20, 200, and 2,000 U/ml of rINFγ in complete medium were added to each well (final cell concentration was 3×10^5 /ml and that of rINFγ was 0, 1, 10, 100, and 1,000 U/ml) and incubated in a CO_2 incubator at 37°C. After 18 hr of rINFγ treatment (day 0), each culture was inoculated with 20 μl of cell-free HTLV-III. Fresh culture medium without rINFγ was changed on days 3 and 5. On days 3, 5 and 7, cell numbers were counted by the Trypan blue dye exclusion method.

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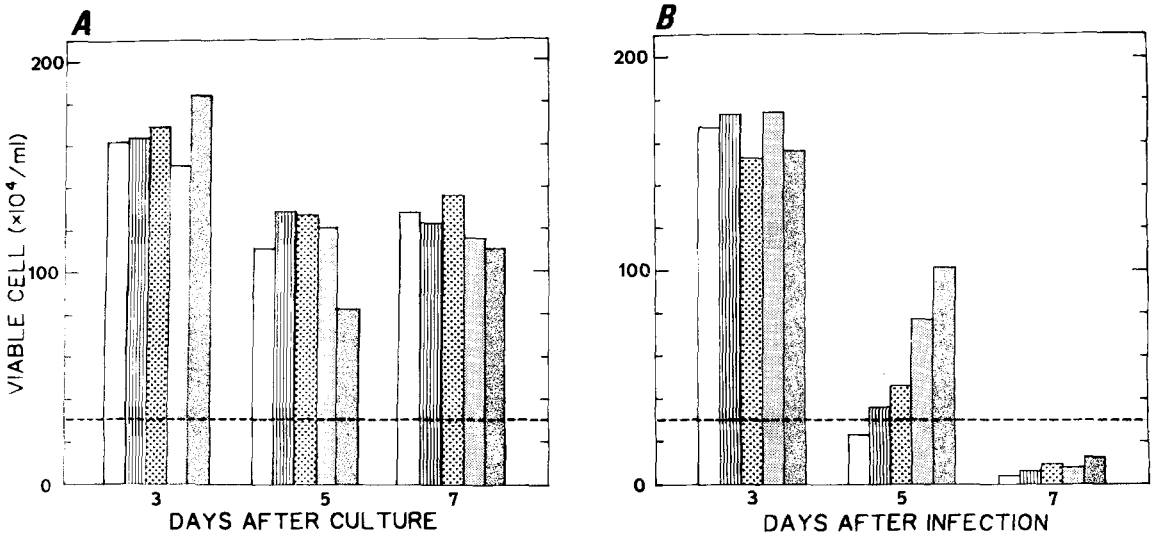

FIGURE 1 – (*a*) Effect of rINFγ on growth of MT-4 cells; and (*b*) inhibition of cytopathic effect of HTLV-III by rINFγ. MT-4 cells were treated with 0 (□), 1 U/ml (▥), 10 U/ml (▩), 100 U/ml (▨), or 1,000 U/ml (▩) of rINF. Infection with HTLV-III was done 18 hr after INF treatment, and the number of cells was determined 3, 5 and 7 days after infection by the Trypan blue dye exclusion method.

Immunofluorescence (IF) technique

The induction of HTLV-III-specific antigens was assessed by the indirect immunofluorescence technique using HTLV-III-seropositive human serum. On days 3, 5, and 7 after infection, cells were smeared, dried and fixed with cold methanol for 3 min. Fixed cells were then incubated with 1:1,000 diluted human anti-HTLV-III-positive serum (IF titer; 1:4,096) for 30 min at 37°C, and the preparation was washed for 15 min with phosphate-buffered saline (PBS). Fluorescein-isothiocyanate-conjugated anti-human IgG (Dakopatts, Copenhagen, Denmark) was applied, and the preparation incubated for 30 min at 37°C and washed again with PBS. Fluorescent cells were examined with a fluorescence microscope and the percentage of the IF-positive cells was calculated.

Plaque assay

Viral preparations for plaque assay were obtained from 4-day-old cultures of HTLV-III-infected MT-4 cells treated with rINFγ at concentrations of 1, 10, 100, and 1,000 U/ml. To obtain MT-4 cell-monolayers in culture vessels, 1 ml of 50 μg/ml of poly-L-lysine (PLL; Mr 120,000, Sigma, St. Louis, MO) was dropped into 35-mm polystyrene tissue culture dishes. Dishes were incubated for 1 hr at room temperature, then PLL-coated dishes were washed 3 times with PBS. MT-4 cells (1.5 ml of 150 × 10^4 cells/ml) were then placed into each PLL-coated dish and incubated for 1 hr at room temperature. The dishes were gently washed twice with PBS to remove unbound cells. Then, 100 μl of viral preparations were slowly added to dishes and incubated for 1 hr at room temperature. After adsorption of virus, 1 ml of the agarose overlay medium consisting of RPMI 1640 medium with 10% FCS, antibiotics and 0.6% agarose (Sea Plaque Agarose, Marine Colloids, Rockland, ME) was poured into each dish. The dishes were incubated in a CO_2 incubator at 37°C for 3 days and 1 ml of agarose overlay medium containing neutral red was added. The dishes were incubated at 37°C for 3 more days and visible plaques were counted.

RESULTS

Inhibitory effect of rINFγ on HTLV-III-induced cell damage in MT-4 cells

Growth of MT-4 cells was not significantly affected by the treatment with 1-1000 U/ml of rINFγ as compared to untreated control (Fig. 1*a*). However, in MT-4 cells infected by HTLV-III, numbers of viable cells were 24, 39, 43, 80, and 102 × 10^4 /ml on day 5 after infection when treated with 0, 1, 10, 100, and 1,000 U/ml of rINFγ, respectively (Fig. 1*b*). The viability of MT-4 cells infected by HTLV-III was 45% on day 5 after infection, but those of the cells treated with 100 and 1,000 U/ml of rINFγ were 94 and 96%, respectively (data not shown). The number and viability of infected cells then decreased rapidly even in the cultures treated with higher concentrations of the drug, reaching the same level as that of untreated control cultures on day 7.

Inhibition of HTLV-III-specific antigen induction in MT-4 cells by rINFγ

On day 5 after infection, the percentages of IF-positive cells in the MT-4 population infected with HTLV-III were 70, 58, 32, 16, and 12% when treated with 0, 1, 10, 100, and 1,000 U/ml of rINFγ, respectively (Fig. 2). However, this suppressive effect of rINFγ was not very significant on day 7 after infection. In order to exclude the possibility of inactivation of rINFγ during culture, fresh rINFγ was added into cultures on day 3 when one-half of the medium was changed, and the presence and manifestations of viral antigen in MT-4 cells infected by HTLV-III were investigated in the same manner as above. The results indicate that the suppressive effect of rINFγ was not

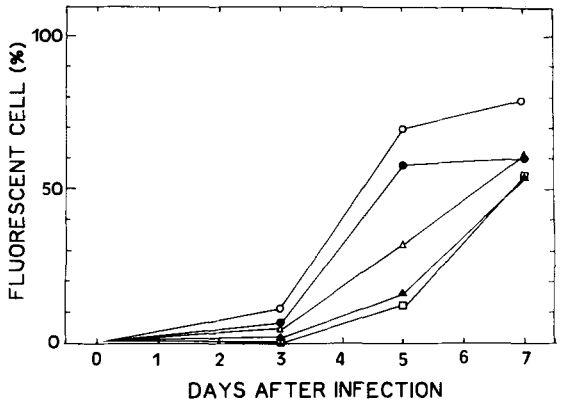

FIGURE 2 – Inhibition of HTLV-III-specific antigen induction in MT-4 cells by rINFγ. Cells were treated with 0 (○), 1 U/ml (●), 10 U/ml (△), 100 U/ml (▲) or 1,000 U/ml (□) of rINFγ. At the indicated times after infection frequency of antigen-positive cells was determined by indirect immunofluorescence.

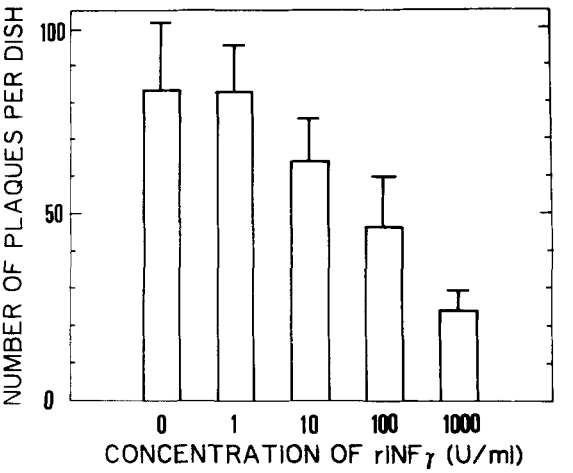

FIGURE 3 – Suppressive effect of rINFγ on HTLV-III replication in MT-4 cells determined by a plaque assay. Results are expressed as number of plaques per dish (±1SD). Details are given in "Material and Methods".

significant on day 6 after infection, although it again inhibited the growth of HTLV-III by day 3 (data not shown).

Suppressive effect of rINFγ on HTLV-III replication

To estimate HTLV-III replication in a more quantitative way, the number of infectious virus particles released from 4-day-old cultures of HTLV-III-infected MT-4 cells treated with various concentrations of rINFγ was counted by means of the plaque-forming assay. The number of plaques per dish decreased in a drug-dose-dependent manner (Fig. 3). About 50% of HTLV-III replication was inhibited by 100 U/ml and 80% was inhibited by 1,000 U/ml of rINFγ.

DISCUSSION

The clinical manifestations of AIDS occur when the immune system is critically depleted of the $OKT4^+$ (helper/inducer) subset of T-lymphocytes (Popovic *et al.*, 1984*a*; Klatzmann *et al.*, 1984*a*) through infection with the human T-lymphotropic retrovirus HTLV-III/LAV (Popovic *et al.*, 1984*b*; Barré-Sinoussi *et al.*, 1983). The lack of helper/inducer T-lymphocyte function eventually results in an immune deficiency state which leaves the host unprotected against environmental opportunistic microorganisms.

Interferon has been reported to inhibit many animal retroviruses, including murine leukemia viruses, murine sarcoma virus and feline leukemia virus (Pitha *et al.*, 1982; Aboud and Hassan, 1983; Sen *et al.*, 1984). These effects are believed to be mediated in part by some critical alterations of viral transcription, translation, assembly and release in the host cells (Pitha *et al.*, 1982; Aboud and Hassan, 1983). Gamma interferon is one of the antiviral/immunomodulatory agents which are capable of enhancing monocyte/macrophage Ia and FcR expression as well as monocyte-mediated cytotoxicity, release of interleukin-1, and priming for tumoricidal activity (Murray *et al.*, 1984). Moreover, HTLV-III can also infect monocyte/macrophages. Experiments on antiviral effects of rINFγ in a monocyte/macrophage system would thus be interesting and appropriate.

Our system for quantitating HTLV-III has allowed us to study the effect of rINFγ in great detail. In this *in vitro* work, rINFγ exhibits a dose-related suppressive effect on HTLV-III infection in MT-4 cells until day 5 after infection. Inhibitory activity on HTLV-III replication is apparent at concentrations between 10 and 1000 U/ml, approximately 80% of HTLV-III replication being inhibited by 1,000 U/ml. Such concentrations of rINFγ do not interfere at all with MT-4 cell growth. We thus believe that rINFγ specifically inhibits HTLV-III replication *in vitro* in a different way from some non-specific drugs which are suppressive through their inhibitory activity on host-cell DNA (data not shown). Moreover, the decrease in the number of plaques was drug-dose dependent. These data clearly show that rINFγ suppresses HTLV-III replication in infected MT-4 cells. However, rINFγ did not show a complete inhibitory effect on HTLV-III infection, especially at day 7 after infection. Initially we thought that this was simply due to medium change which caused dilution or inactivation of rINFγ. Further studies, however, revealed that this was not the case since re-addition of fresh rINFγ did not essentially change the results. At present, we do not know whether this is due to an alteration of cellular responsiveness to rINFγ during culture after HTLV-III/LAV infection. With our experimental system it is also possible that the antiviral effect of rINFγ reported here is indirect and is mediated by an effect of HTLV-I on MT-4 cells. Experiments using normal peripheral blood lymphocytes as target cells to demonstrate an antiviral effect of rINFγ might be important, too, and another quantitative assay using normal peripheral blood lymphocytes should be developed to this effect.

Ho *et al.* (1985) have reported that rINFαA has an inhibitory activity against HTLV-III replication in normal lymphocytes. Completely inhibitory effects against the virus are seen at doses between 256 and 1,024 U/ml when RT-activity is measured. Such inhibitory con-

centrations as well as those obtained by us with rINFγ and reported here, are within the range that may be achievable in the blood of patients after infection.

Several trials of interferon in the clinical therapy of AIDS have given some results (Krown *et al.*, 1983; Groopman *et al.*, 1984; Gelmann *et al.*, 1985). However, these trials have generally been carried out at a late stage of the disease, when significant antiviral effects of the substance may be difficult to ascertain. Trials performed at earlier stages of the disease, or trials associating interferon with other antiviral agents or immunomodulators, may be necessary to demonstrate its antiviral activity. Moreover, much remains to be learned concerning the *in vivo* fate of these compounds and their precise *in vivo* potential. In this respect, it is emphasized that HTLV-III has been detected in and recovered from the central nervous system, and that it is essential to determine whether interferon crosses the blood-brain barrier.

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