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I. INTRODUCTION. Recently, there has been interest in the production of IFNs during the course of HIV infections, and in the possible therapeutic use of IFNs in AIDS-associated Kaposi sarcoma (Krown et al, 1990) or in AIDS. These were the subjects of two meetings held in New York City in 1990, partially on the basis of a report that treatment of clinical AIDS with IFN $\alpha$  is effective, and a preliminary report that long-term treatment of HIV carriers prolonged their asymptomatic incubation period (Lane et al, 1990; Rivero et al, 1990).

IFNs have a significant inhibitory effects on replication of retroviruses, including lentiviruses (Friedman and Pitha, 1984). Two major mechanisms of action have been identified, both relating to virus assembly: (1) in the case of most murine leukemia viruses (MLVs) and mouse mammary tumor virus, IFN caused an inhibition of virus budding. This lead to the accumulation of large numbers of mature particles at or below the plasma membrane (Chang et al, 1977). (2) However, in infections with AKR- and Moloney-MLV, and VSV, the latter a rhabdovirus, altered virus particles were produced, deficient in gp69/71 in MLVs, or G-protein in VSV (Friedman et al, 1980). Because of the deficiency in glycoprotein the released virus was defective in infectivity. This may be related to an inhibition of the transport of viral glycoproteins from the trans Golgi to the plasma membrane (Singh, VK et al, 1988), that correlated with a significant IFN-induced rise in pH of the normally highly acidic Golgi. Both effects of IFN occurred late in virus replication; indeed, in most studies of IFN effects on retroviral replication, there were no significant changes in virus RNA or protein synthesis and processing (Friedman and Pitha, 1984).

In view of the large number of publications on HIV and HIV-related disease, reports on the mechanisms of antiviral effects of IFN treatment on HIV infection are relatively sparse (see Kornbluth et al, 1990 for a review of IFN effects on HIV replication). These indicate that the effect of IFN treatment on HIV replication in a line of T-lymphocytes resembled the major reported action on MLV and MMTV replication, inhibition of virus morphogenesis (Poli et al, 1989). In the case of infected macrophages, IFN $\alpha$  action differed both qualitatively and quantitatively from that seen in T-cells, and was more complex. Treatment before HIV infection blocked the production of HIV-cDNA (Kornbluth et al, 1990; Gendelman et al, 1990a), although there was one previous report that IFN treatment had no effect under these conditions (Hartshorn et al, 1987). IFN $\alpha$  treatment with or without AZT soon after exposure to HIV, such as in needle sticks, may therefore offer the best chance we have of preventing HIV infections at the present time.

Our group has shown, moreover, that IFN $\alpha$  treatment of macrophages chronically infected with the Ada strain of HIV had two distinct inhibitory effects: the production of HIV-mRNA was inhibited; and, there was an increase in production of virus particles defective in infectivity (Gendelman et al, 1990a). Treatment with IFN $\alpha$  also modified the major cytopathic effect of HIV infection on macrophages, the formation of multinucleated giant

cells (Gendelman et al, 1990a). One of the major aims of this application is to study the molecular mechanisms by which these inhibitory actions of recombinant (r)IFN $\alpha$  are effected. Study of the activity of IFNs on HIV-I may yet define a therapeutic role for them in AIDS, or alternatively suggest sites of vulnerability of HIV to other agents.

There is perhaps more familiarity with IFN $\alpha$  production in AIDS, since significant titers of IFN were reported by us to be present in serum of patients with active or developing disease, even before the etiologic agent in AIDS had been uncovered (DeStefano et al, 1982). The IFN present may account for some of the symptoms characteristic of AIDS such as fever, muscle pain, wasting, and malaise (Krown et al, 1990). Endogenous IFN production might also contribute to the establishment of latency in HIV infection. We and others reported the dire prognosis of patients developing circulating titers of IFN; they have a very high probability of going on to active disease within 6 to 12 months (Goedert et al, 1989). In addition, the IFN $\alpha$  produced by HIV-infected patients is unusual, being partially labile at pH2, unlike other IFN $\alpha$  subtypes (DeStefano et al, 1982). A recent paper has suggested that acid-lability is not an inherent property of a similar IFN found in patients with systemic lupus erythematosus, SLE (Yee et al, 1989). The aims of this application include study of the nature of this IFN, its cellular source, properties, and role, together with that of other cytokines in the pathogenesis of AIDS.

The IFN $\alpha$  produced by HIV-infected cells is unlikely to be a product of the macrophage alone, because potent inducers of IFN $\alpha$  production were significantly less effective in HIV-infected, purified macrophages than in controls. This is a specific inhibition in the induction of IFN $\alpha$ ; production of other IFNs and cytokines by macrophages was not downregulated by HIV infection (Gendelman et al, 1990b).

Although we have found that in vitro HIV-infected, highly purified cultures of lymphocytes or macrophages did not produce IFN (Gendelman et al, 1990a), cultures with mixtures of the two cell types produced significant titers of IFN $\alpha$  (Szebeni et al, 1989). We wish to find which particular cell is responsible for the production of IFN $\alpha$ , and what is the nature of the interaction between macrophages and lymphocytes. Interestingly, restriction of the replication of visna-maedi, a lentivirus, in ovine lung macrophages by virus-induced IFN required the presence of lymphocytes (Narayan et al, 1985).

Probably because the only source of the IFN found in AIDS has hitherto been the serum of infected patients, very little is known about its properties. Our preliminary finding was that the culture fluids containing IFN produced by HIV-infected cells in vitro inhibited HIV replication to a significantly lesser extent than did equivalent titers of rHuIFN $\alpha$ . This may be an inherent property of the HIV-induced IFN, or be due to the presence of additional cytokines in the culture fluids. This finding could account for the coexistence in active AIDS of both progressive infection and significant titers of a putative HIV inhibitor, IFN. Understanding

of the mechanisms of IFN production and action in the course of AIDS might lead to harnessing the potentially significant antiviral activities of both endogenous and exogenous IFN.

## II. RESULTS.

### A. Biological actions of IFN.

1. Activity on HIV-infected macrophages. a. Effect of early treatment with IFN. In cells treated with IFN before or at the time of HIV infection, there was marked inhibition of virus replication with as little as 5 int'l units(IU)/ml. With higher concentrations the inhibition was total, so that no proviral DNA was detectible by PCR 7d after infection (Fig 1, lane 5). Polymerase chain reaction methodology plays a critical role in this project. In the past three years, we have developed new PCR technology (Lowe et al., 1990), designed and used PCR primers for the study of gene expression in several systems (Jacobsen et al., 1989; Gendelman et al, 1990b; Bickel et al., 1990; Diamond et al., 1990; Rimoldi et al., 1990; Majde et al., 1990), and recently explored regulation and expression of cytokines and HIV-1 in HIV-infected cells (Gendelman et al., 1990 A&B; Kalter et al., 1991). We have also developed a new PCR computer program that is in wide-spread use. Because of our experience in virological, PCR, and IFN methodologies, we feel we have the expertise necessary to carry out the proposed research.

b. Inhibition of HIV-mRNA in chronically infected cells. In cultures of macrophages infected with HIV for 7 days, and then treated with IFN, there was a significant dose-dependent inhibition of viral gene expression as early as 5h after initiation of IFN treatment. This inhibition continued as long as IFN was present. Although the IFN treatment had no effect on the levels of HIV proviral DNA (Fig 1, lanes 3&4), there was a marked reduction in HIV-mRNA, p24, and RT in treated cells (Fig 2A&B).

c. Inhibition of the infectivity of progeny virus in chronically infected cultures. An additional antiviral effect was observed when macrophages infected for 7 days were treated with IFN. When comparing yields of infectious HIV cultured from IFN treated and control cells, adjusted to contain equal levels of RT activity, the early progeny virus particles from the treated cultures were reduced 100- to 1,000-fold in infectivity (Fig 3). This effect was in some respects similar to that observed in MLV- or VSV-infected cells treated with IFN, where the infectivity of progeny particles was markedly reduced due to decreased levels of glycoprotein incorporated into the virion (Friedman et al, 1980).

In studies of the formation of VSV deficient in glycoprotein (G), we have found that production of G was inhibited less than 5-fold in IFN-treated cells, but transport of G from the trans Golgi to the plasma membrane was defective, resulting in its retention in the Golgi (Singh, VK et al, 1988). The 50- to 500-fold reduction in infectious VSV yields observed might have been a consequence of this inhibition of G transport. This effect of IFN was potentiated by primary amines such as chloroquine, dansylcadaverine, and ammonium chloride, that raised intra-cellular pH (Maheshwari et al,

1991); furthermore, IFN treatment induced a significant rise in the pH of the Golgi apparatus that could be localized using DAMP (Fig 4A&B).

Since IFN and the amines were synergistic in their effect on intracellular pH, they would appear to have differing mechanisms of action. Indeed, the rise in pH induced by IFN, but not by amines, was blocked by amiloride, an inhibitor of the  $\text{Na}^+/\text{H}^+$  antiporter; moreover, concentrations of IFN that alkalinized the Golgi increased the intracellular  $\text{Na}^+$  level, also suggesting an alteration in the antiporter (Maheshwari et al, 1991).

d. Recision of in vitro HIV-induced cytopathic effects(CPE).

HIV-infected macrophages sometimes undergo extensive multinucleated giant cell formation. In our system, elutriated monocytes infected with the Ada strain of HIV-I, 30 to 50% of the culture, depending on the multiplicity of infection, was involved in this CPE by 7-10d after infection (Fig 5, center), although minimal giant cell formation was observed in uninfected, cultured monocytes (Fig 5, left). Addition of IFN (500 IU/ml) at 7d after infection altered this CPE, so that by 14d the cultures differed only marginally from uninfected cells (Fig 5, right).

2. Effect on T-cells. IFN treatment of HIV-infected T-lymphoblasts had no significant effect on proviral DNA (Fig 1, lanes 1&2), protein, or RNA synthesis; however, as in the case of many MLV infections treated with IFN, extracellular virus production was inhibited, as indicated by a decrease in extracellular RT. Intracellular RT was, however, increased. By analogy with the findings in MLV infections (Friedman & Pitha, 1984) and the results of others (Poli et al, 1989), these experiments suggested an inhibition of HIV morphogenesis.

B. IFN production

1. IFN production by HIV-infected cells in vitro. a. Inhibitory effect of HIV infection on IFN $\alpha$  production. Purified macrophages produced high titers of IFN $\alpha$  when infected with viruses or treated with the IFN inducer, polyinosinic-polycytidylic acid (poly IC). Infection with HIV (culture 50 % productively infected) almost completely blocked this response to virus infections or to poly IC (Fig 6); however, HIV infection did not inhibit the ability of the monocytes to produce mRNAs for IFN $\beta$  or  $\omega$ , or the cytokines IL-1 $\beta$ , IL-6, or TNF $\alpha$  (Fig 7A&B). This effect of HIV infection thus appeared to be specific for IFN $\alpha$ .

b. Cell type producing IFN. No IFN $\alpha$  was produced by highly purified lymphoblasts or macrophages at any time up to three weeks after in vitro infection with each of 15 strains of HIV (Gendelman et al, 1990a); however, macrophages infected with HIV for 7d, to which uninfected lymphocytes were then added (final mixture was 90% macrophages) produced titers of IFN $\alpha$  ranging as high as 500 to 1000 IU/ml within 14h. Capobianchi et al (1988) also reported that more than one cell type was required for in vitro IFN $\alpha$  production.

c. Lack of anti-HIV activity of IFN $\alpha$  produced in cells infected in vitro. The IFN $\alpha$  produced by HIV-infected cultures (see the preceding paragraph) had antiviral activity against VSV and EMC virus in a conventional CPE inhibition assay, and was neutralized

by NIH reference anti-IFN $\alpha$  antibody. When tested for anti-HIV activity, however, this IFN $\alpha$  had less than 1/500th of the inhibitory effects (as described in section A1) of comparable concentrations in int'l units of rIFN $\alpha$  on the replication of HIV in macrophages.

2. Nature of IFN induced by HIV infection in vivo. HIV-infected patients often have in their serum a pH 2 labile IFN $\alpha$  (DeStefano et al, 1982). Because of the report that in SLL patients, who produce a similar IFN, acid-lability is not an intrinsic property of the IFN, being imparted by a serum factor (Yee et al, 1989), we characterized further the serum IFN of HIV-infected patients, adsorbing it to an anti-IFN $\alpha$  antibody affinity column. All of the serum antiviral activity was removed by the column, which was then washed with saline, and the IFN eluted with 4M guanidine. The IFN eluting in early fractions (50%) was a conventional IFN $\alpha$ , completely resistant to pH 2 treatment; the IFN $\alpha$  eluted in late fractions, however, was almost as acid-sensitive as IFN $\gamma$ , with 90% of the antiviral effect inactivated.

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PRODUCTION OF LABILE ALPHA INTERFERON BY AIDS PATIENTS

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Publications:

Gendelman, H.E., Baca, L.M., Turpin, J., Kalter, D.C., Hansen, B., Orenstein, J.M., Dieffenbach, C.W., Friedman, R.M., Meltzer, M.S. (1990) J. Immunol. 145:2669.

Gendelman, H.E., Friedman, R.M., Joe, S., Baca, L.M., Turpin, J.A., Dveksler, G., Meltzer, M.S., Dieffenbach, C.W., (1990) J. Exptl. Med. 172:1433.

Kalter, D.C., Nakamura, T., Turpin, J., Baca, L.M., Hoover, D.L., Dieffenbach, C.W., Ralph, P., Gendelman, H.E., Meltzer, M.S. (1991) J. Immunol. 146:34.



Figure 1. Effect of IFN on levels of proviral DNA in HIV-infected lymphoblasts and monocytes. Cell lysates of HIV-infected lymphoblasts and monocytes were extracted twice with phenol and chloroform/isoamyl alcohol and the DNA precipitated with ethanol. Polymerase chain reaction amplification of HIV-specific gag DNA with nucleotide primers from the 5'LTR and gag genes and Taq polymerase was performed with an automatic cyclor. The products of 40 cycles were analyzed by Southern blot hybridization with a radiolabeled HIV-specific DNA probe after agarose gel electrophoresis. Autoradiographs show samples from 7 day HIV-infected lymphoblasts treated at the time of infection with medium (lane 1) or 500 IU/ml IFN (lane 2), from 14 day HIV-infected monocytes treated 7 days after infection with medium (lane 3) or 500 IU/ml IFN (lane 4), and from 14 day HIV-infected monocytes treated at the time of infection with 500 IU/ml IFN (lane 5). The lambda DNA negative and HXB2 proviral DNA positive controls are shown in lanes 6 and 7, respectively.

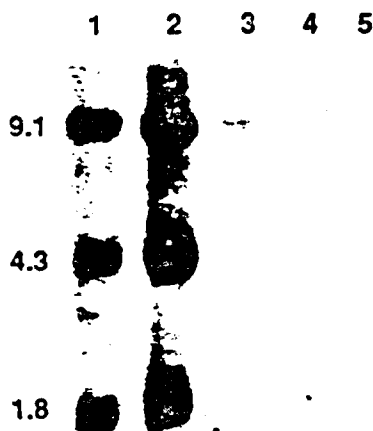


Figure 2A. Effect of IFN on HIV-specific mRNA in infected monocytes. PBMC purified to >98% monocytes and cultured 7 days as adherent monolayers in medium with human serum and MCSF were exposed to the HIV isolate, ADA, at a multiplicity of infection of 0.01 tissue culture ID<sub>50</sub>/cell. Seven days after infection, 500 IU/ml rIFN- $\alpha$  were added and maintained at this concentration throughout the culture interval. Cell lysates of HIV-infected monocytes were extracted with acidic guanidinium isothiocyanate/phenol/chloroform and the RNA precipitated with isopropanol. RNA was analyzed by Northern blot hybridization with a radiolabeled HIV-specific LTR probe after agarose gel electrophoresis under denaturing conditions. Autoradiographs show samples from HIV-infected monocytes 7 days (lane 1) or 14 days (lane 2) after infection, and HIV-infected monocytes treated at 7 days with 500 U/ml IFN and cultured with IFN for an additional 7 days (lane 3). RNA from cell lysates of uninfected monocytes treated with medium (lane 4) or 500 U/ml rIFN are shown in lane 4 and 5, respectively.

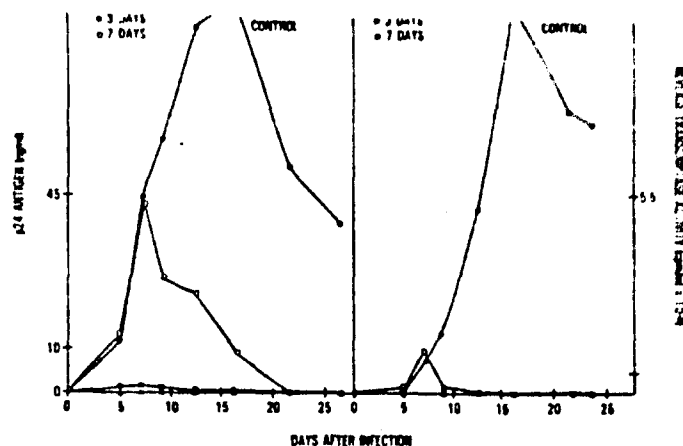


Figure 2B. Effect of IFN on replication of HIV in monocytes at various times after infection. PBMC purified to 98% monocytes and cultured 7 days as adherent monolayers in medium with human serum and MCSF were exposed to the HIV isolate, ADA, at a multiplicity of infection of 0.01 tissue culture ID<sub>50</sub>/cell. Seven days after infection, 500 IU/ml rIFN- $\alpha$  were added and maintained at this concentration throughout the culture interval. Levels of p24 Ag in culture fluids (left panel) were determined by ELISA. Levels of RT activity in culture fluids are shown in the right panel.

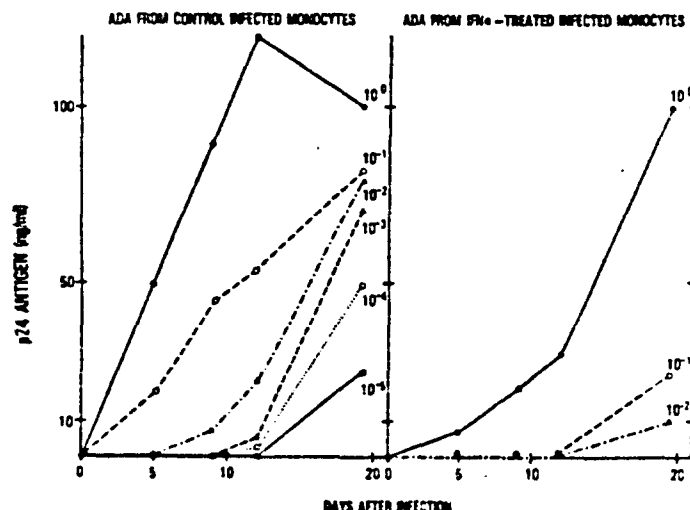


Figure 3. Effect of IFN on infectious progeny virus from HIV-infected monocytes. PBMC purified to 98% monocytes and cultured 7 days as adherent monolayers in medium with human serum and MCSF were exposed to the HIV isolate, ADA, at a multiplicity of infection of 0.01 tissue culture ID<sub>50</sub>/cell. Seven days after infection, 500 IU/ml rIFN- $\alpha$  were added and maintained at this concentration throughout the culture interval. At 55 hr, virus in culture fluids from control and IFN-treated infected cells was pelleted by ultracentrifugation and adjusted to equal levels of RT activity. Dilutions of these viral inoculum were added to new monocyte target cells. All cultures were refed with fresh medium every 2 to 3 days and the culture fluids assayed for p24 Ag.

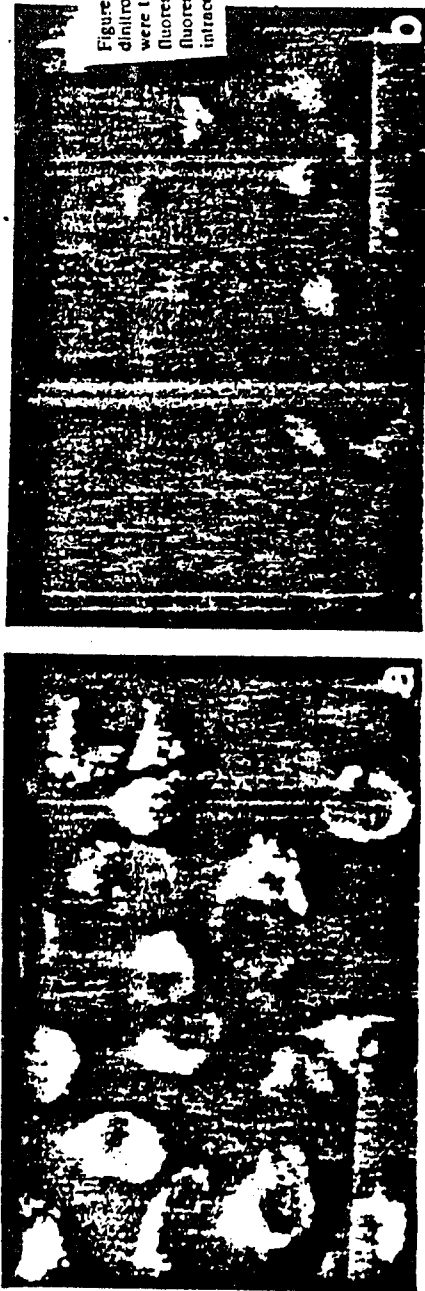


Figure 4. Effect of Interferon (IFN- $\gamma$ ) on Intracellular distribution of 3 (2,4-dinitroanilino)-3'-amino-N-methylpropylamine (DAMP) in IgG cells. IgG cells were treated with MoIFN- $\gamma$  (30 IU/ml) for 16 hr. DAMP was localized by fluorescence microscopy using mouse anti-DNP antibody, followed by a fluorescein-coupled rabbit anti-mouse globulin antibody. DAMP accumulation in intracellular acidic compartments. a) untreated cells; b) IFN-treated cells.



Figure 5. Effect of IFN on cytopathic effects in HIV-infected monolayers. PBMC purified to 98% monocytes and cultures 7 days as adherent monolayers in medium with human serum and MCSF were exposed to the HIV isolate, ADA, at a multiplicity of infection of 0.01 tissue culture ID<sub>50</sub>/cell. Seven days after infection, 500 IU/ml rIFN- $\alpha$  were added and maintained at this concentration throughout the culture interval. Wright-stained monolayers represent: uninfected monocytes at 7 days (left panel), HIV-infected monocytes at 7 days with typical HIV-induced multinucleated giant cells (middle panel), and HIV-infected monocytes treated at 7 days with 500 IU/ml IFN- $\alpha$  and cultured for an additional 7 days (right panel).

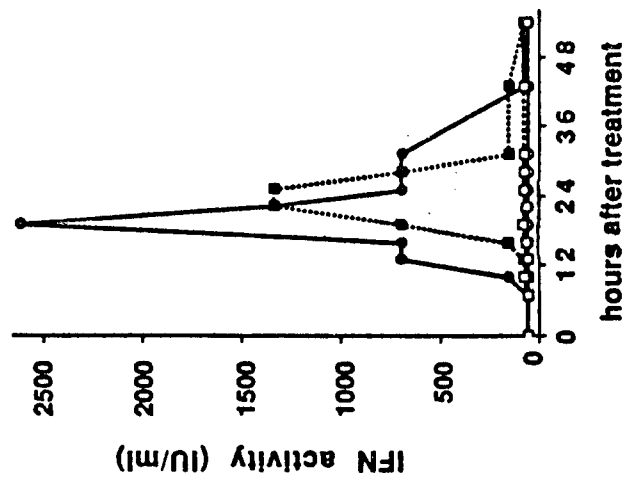


Figure 6. Time course for IFN production by HIV-infected monocytes treated with poly(I)-poly(C) and NDV. Monocytes cultured 7 days as adherent monolayers were exposed to HIV at an MOI of 0.01 infectious virus/target cell. Two weeks after infection, 100  $\mu$ g/ml poly(I)-poly(C) (circles) or NDV (squares) at an MOI of 1 infectious virus/target cell was added to HIV-infected (open symbols) and uninfected control (closed symbols) cultures for 4 hours. All cultures were washed free of poly(I)-poly(C) and NDV and refed with fresh medium. IFN activity in culture fluids was assayed at 3 hour intervals by inhibition of murine encephalomyocarditis virus-induced cytopathic effects in human fibroblasts. Each data point represents the mean of duplicate determination in one of four replicate experiments.

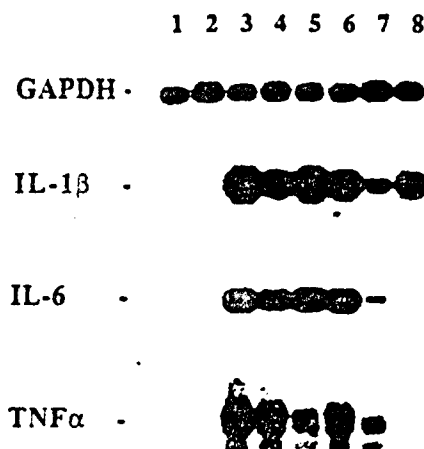


Figure 7A. Induction of IFN- $\omega$  and IFN- $\beta$  mRNA in HIV-infected monocytes by poly(I)-poly(C). Monocytes cultured 7 days as adherent monolayers were exposed to HIV at an MOI of 0.01 infectious virus/target cell. Two weeks after infection, 100  $\mu$ g/ml poly(I)-poly(C) was added to HIV-infected and uninfected control cultures for 4 h. All cultures were washed and refed with fresh medium. RNA from cell lysates was extracted and mixed with antisense primers. After reverse transcription, cDNA was amplified by PCR and the products of 25 cycles were analyzed by Southern blot hybridization with a IFN- $\omega$  and IFN- $\beta$  specific probe. Coupled reverse transcription/PCR amplification products from cell lysates of monocytes at various times after poly(I)-poly(C) treatment for uninfected cells are shown in lanes 1 (0 h), 3 (8 h), 5 (12 h), and for HIV-infected cells in lanes 2 (0 h), 4 (8 h), 6 (12 h), 7 (24 h), and 8 (48 h).

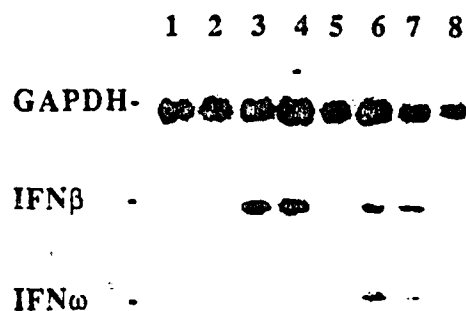


Figure 7B. Induction of cytokine mRNA in HIV-infected monocytes by poly(I)-poly(C). Monocytes cultured 7 d as adherent monolayers were exposed to HIV at an MOI of 0.01 infectious virus/target cell. Two weeks after infection, 100  $\mu$ g/ml poly(I)-poly(C) was added to HIV-infected and uninfected control cultures for 4 h. All cultures were washed and refed with fresh medium. RNA from cell lysates was extracted and mixed with antisense primers. After reverse transcription, cDNA was amplified by PCR and the products of 25 cycles were analyzed by Southern blot hybridization with cytokine-specific probes. Coupled reverse transcription/PCR amplification products from cell lysates of monocytes at various times after poly(I)-poly(C) treatment for uninfected cells are shown in lanes 1 (0 h), 3 (8 h), 5 (12 h) and for HIV-infected cells in lanes 2 (0 h), 4 (8 h), 6 (12 h), 7 (24 h), and 8 (48 h).