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Antiviral activity of mismatched double-stranded RNA against human immunodeficiency virus *in vitro**

(interferon/T-cell lysis/human T-cell lymphotropic virus III and lymphadenopathy-associated virus replication)

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ABSTRACT **The biological response modifier $r(I)_n \cdot r(C_{12}\text{-}U)_n$, referred to here as mismatched double-stranded (ds) RNA, was examined for antihuman immunodeficiency virus (HIV) activity *in vitro* because of its known antiviral activity and ability to induce interferon (IFN) in other biological systems [Carter, W. A., Strayer, D. R., Hubbell, H. R. & Brodsky, I. (1985) *J. Biol. Response Modif.* 4, 495–502]. We found that cultures of the highly HIV-permissive T-cell line C3 were afforded significant protection from HIV infection when incubated in growth media supplemented with mismatched dsRNA at 10–50 μg/ml prior to virus challenge. Similar results were obtained at 50 μg of mismatched dsRNA per ml in cultures of the T-lymphoblastoid cell line CEM. Infections were monitored by indirect immunofluorescence of cells for viral p24 antigen expression, reverse transcriptase activity in culture fluids for virus production, and vital dye uptake for cytopathic effect. Antiviral activity was increased by the continued presence of mismatched dsRNA in cultures following virus challenge. A one-time exposure to mismatched dsRNA (50 μg/ml) provided greater antiviral activity than either a one-time exposure to recombinant IFN-α [250 international units (IU)/ml], IFN-β (250 IU/ml), or IFN-γ (50 IU/ml) in cultures of CEM cells, or a one-time exposure to a combination of all three IFNs (150 IU each per ml) in cultures of C3 cells. Mismatched dsRNA at 50 μg/ml had no effect on cell division, RNA and protein synthesis, or virus replication in all T-cell lines examined. A clear distinction between the activities of mismatched dsRNA and IFN was the ability of IFN to suppress the *in vitro* replication of HIV that occurred at IFN concentrations (150 IU each of α, β, and γ per ml) that provided less antiviral activity than mismatched dsRNA (50 μg/ml). The results of these *in vitro* studies suggest a potential therapeutic value for mismatched dsRNA in the treatment of acquired immunodeficiency syndrome (AIDS).**

Human immunodeficiency virus (HIV), also known as human T-cell lymphotropic virus III (HTLV-III) or lymphadenopathy-associated virus (LAV), is a highly variable type D retrovirus recently identified as the etiologic agent of acquired immunodeficiency syndrome (AIDS) (1–3). A distinguishing feature of the virus is its selective cytotoxicity for helper T lymphocytes (4). Infection *in vivo* is manifested by severe and diverse aberrations of the immune system, primary among these being a decreased T-helper/T-suppressor cell ratio (5). In addition, monocytes have been identified as secondary targets for HIV infection and may be responsible for the frequent encephalopathy associated with AIDS (6). Other immune dysfunctions associated with AIDS, but of unknown relationship to direct virus infection, include impaired natural killer cell activity (7–9), polyclonal B-cell activation (10, 11), and a wide range of serological abnormalities (5).

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The first generation, specific anti-AIDS chemotherapeutic agents have focused on inhibition of HIV replication with the anticipation that an effective drug would protect uninfected HIV target cells from a progressive destruction of the host immune system (12). One such candidate for the treatment of AIDS is interferon (IFN). The three molecular forms of IFN (i.e., α, β, and γ) are well known for their antiviral, antineoplastic, and immunomodulatory activities (13, 14). It is reasonable to hypothesize that the biological responses elicited by IFN *in vivo* could modulate the natural progression of AIDS by inhibiting HIV replication, suppressing AIDS-associated neoplasms, and facilitating the reconstitution of impaired immune functions. Initial evidence for the potential efficacy of IFN in the therapy of AIDS came from the finding that antiserum to human IFN-α facilitates the isolation of HIV from peripheral blood mononuclear cells (PBMCs) of AIDS patients *in vitro* (1). Additional evidence came from the finding that recombinant IFN-αA can protect normal PBMCs from infection with HIV *in vitro* (15). Recently, several other molecular forms of IFN-α and a preparation of IFN-β were also shown to inhibit the *in vitro* replication of HIV in PBMCs (16).

Double-stranded RNAs (dsRNAs) are inducers of the various molecular forms of IFN, activate certain IFN-associated intracellular mediators, possess immunomodulatory activities and inhibit tumor cell proliferation (17–25). The therapeutic value of dsRNAs as antiviral/anticancer drugs was initially difficult to assess due to the toxicity of the prototype molecule $r(I)_n \cdot r(C)_n$ (23, 26). Later it was found that by introducing a mismatched, non-hydrogen-bonded base residue (uracil) into the polycytidylic acid polymer strand at every twelfth residue, the mismatched dsRNA polymer, $r(I)_n \cdot r(C_{12}\text{-}U)_n$, was rendered sensitive to RNase destruction while retaining the one helical turn required for biological activity (27). This mismatched analogue, commonly known as Ampligen, is much less toxic while retaining the therapeutic properties mentioned above (18–22). These therapeutic properties, combined with a low-toxicity profile, make mismatched dsRNAs important candidates as potential anti-AIDS drugs. In the present study we have assessed this potential *in vitro* and found that mismatched dsRNA of the form $r(I)_n \cdot r(C_{12}\text{-}U)_n$ affords significant protection of T-cells from infection by two HIV isolates, HTLV-III and LAV.

MATERIALS AND METHODS

Cell and Virus Stock Cultures. CEM/LAV (28) and H9/HTLV-III(29) were used as producer cell lines for the AIDS

Abbreviations: HIV, human immunodeficiency virus; dsRNA, double-stranded RNA; IFN, interferon; rIFN, recombinant IFN; PBMC, peripheral blood mononuclear cell; IIF, indirect immunofluorescence; RT, reverse transcriptase; PBS, phosphate-buffered saline; IU, international units; AIDS, acquired immunodeficiency syndrome.

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viral isolates LAV and HTLV-III, respectively. The T-lymphoblastoid cell line CEM (30) and the HTLV-II-transformed umbilical cord blood T-cell line C3 (31) were used as targets for infections. All cultures were maintained in RPMI 1640 medium supplemented with 16% fetal calf serum, 50 μg of gentamicin sulfate per ml, and 2.5 μg of Fungizone (GIBCO) per ml, and incubated at 36°C in culture flasks.

dsRNA and IFNs. The mismatched dsRNA, Ampligen, was provided as a lyophilized powder in a salt buffer by HEM Research (Rockville, MD). Human recombinant IFNs (rIFN) α, β, and γ, having specific activities greater than 10^8 international units (IU) per mg of protein, were obtained from Hoffman–LaRoche. Commercial preparations of human IFN β and γ were obtained from HEM Research and Interferon Sciences (New Brunswick, NJ), respectively. These nonrecombinant IFNs had specific activities greater than 10^6 IU per mg of protein. All IFNs were calibrated on human SG181 foreskin fibroblasts challenged with encephalomyocarditis virus and assayed for viral cytopathic effect as previously described (14, 32). Reference standards were obtained from the World Health Organization (human IFN-α, WHO standard B, 69/19 and human IFN-β, WHO no. G-023-902-527) or the National Institute of Allergy and Infectious Disease (human IFN-γ, National Institutes of Health no. Gg23-901-530).

Assay of Anti-HIV Activity. HTLV-III and LAV preparations for infection studies were obtained by replacing the growth medium on stock H9/HTLV-III or CEM/LAV cultures and allowing fresh virus to accumulate for 2 days prior to harvest and use. Conditioned culture fluids were clarified of cells by low-speed centrifugation and passage through a 0.45-μm cellulose acetate filter. Viral titers are defined in these studies by cpm of reverse transcriptase (RT) activity in culture fluids. Target cells were incubated in 25-cm^2 flasks containing 10 ml of growth medium supplemented with various concentrations of effectors (i.e., dsRNA or IFN). Following the preincubation period, the cultures were inoculated with 5 ml of clarified conditioned H9/HTLV-III or CEM/LAV culture fluid. Virus particles were allowed to adsorb for 5 hr at 36°C, and unadsorbed particles were removed by two washes with 10 ml of RPMI 1640 medium. Cultures were then incubated in 20 ml of fresh growth medium with or without effectors as described. Cell densities were reduced, and media was replaced at given times to avoid overgrowth. Samples for indirect immunofluorescence (IIF), RT assays, cell counts, and neutral red staining were obtained at these times. IIF and RT assays were done as described (33, 34).

Vital Dye Uptake. Cells were collected from suspension by low-speed centrifugation and resuspended in 5 ml of growth medium containing a 1:150 dilution of Finter's stock 2% neutral red dye (35). After a 1-hr staining period at 36°C the cells were washed twice with phosphate-buffered saline (PBS), and the dye was extracted with 5 ml of acidified alcohol (50% ethanol/1% acetic acid). The extracted dye solution, which was linearly proportional to the number of viable cells (1.0×10^5–1.5×10^6) used in these experiments, was quantitated colorimetrically at 540 nm with a Bausch and Lomb model Spectronic 20 spectrophotometer.

Quantitation of Cell Division, RNA and Protein Synthesis, and Virus Replication. C3, CEM, CEM/LAV, CEM/HTLV-III and H9/HTLV-III cell cultures were incubated in the presence and absence of effectors for 4 days with media being replaced on day 2. Cultures were initiated at cell densities that would allow exponential growth for this period. Initial and final cell densities were determined by hemacytometer cell counts. At the end of the 4-day period, conditioned culture fluids were assayed for RT activity, and the cells were divided into two equal cultures; each culture contained 10 ml of fresh growth medium supplemented with the respective effector as before. To one culture was added [5,6-^{3}H]uridine (38.8 Ci/mmol, 1 Ci = 37 GBq; New England Nuclear) and to the other was added ^{3}H-labeled amino acid mix (184 mCi/mg; ICN), both at 20 μCi/ml of culture. After a 4-hr incubation at 36°C the radiolabeled cells were washed excessively with cold (4°C) PBS, suspended in 1 ml of cold PBS and counted in 10 ml of scintillation fluor (3a70B; Research Products International, Mt. Prospect, IL) using a Beckman model LS-6800 liquid scintillation counter.

RESULTS

Mismatched dsRNA and IFN Induced Protection of C3 Cells from HIV Infection. Fig. 1 compares the antiviral activities observed after 7 days in cultures of C3 cells exposed to mismatched dsRNA at 0, 0.4, 2, 10, and 50 μg/ml for 24 hr prior to challenge with the HIV isolate HTLV-III. In the absence of mismatched dsRNA, a dramatic rise in RT activity in culture fluid was associated with a 48% decrease in viable cells which were all positive for HIV p24 antigen expression. Little protection was provided by mismatched dsRNA at 0.4 and 2 μg/ml where RT activities and percent of cells expressing p24 increased, while the number of viable cells decreased to levels approaching those of the control (no mismatched dsRNA). In contrast, cultures pretreated with mismatched dsRNA at 10 and 50 μg/ml had very little RT activity in culture fluids, while virtually no reduction in viable cells was observed and less than 1% of exposed cells were positive for p24 expression.

We next examined the time frames for infection in cultures of C3 cells incubated with mismatched dsRNA (50 μg/ml), an IFN mixture (150 IU each of IFN-α, rIFN-β, and rIFN-γ per ml), or incubated as before plus continual incubation with mismatched dsRNA (50 μg/ml) and compared these treatments with an untreated (control) culture (Fig. 2). The first evidence of HIV infection (>1% of cells p24 positive by IIF) was found at 4 days for the control (5%), 6 days for IFN (10%), 8 days for mismatched dsRNA (10%), and 10 days for cells exposed continuously to mismatched dsRNA (5%). The first detection of significant RT activity (>10^4 cpm/ml) in culture fluids was 6 days for the control (7.0×10^4 cpm/ml), 8 days for IFN (14.5×10^4 cpm/ml), 10 days for mismatched

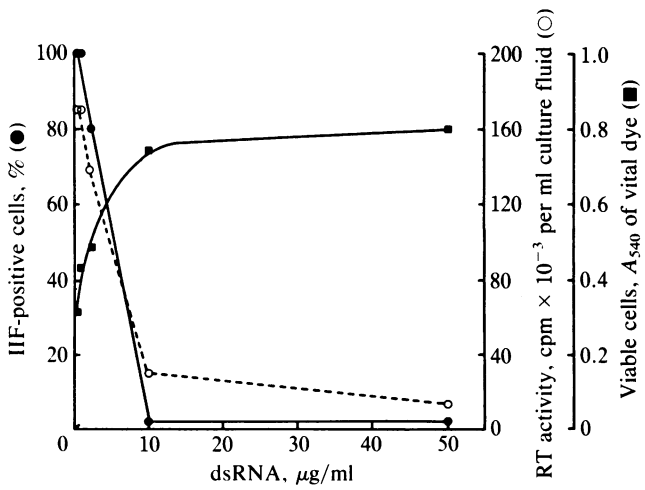

FIG. 1. Dose–response of C3 cells to the anti-HTLV-III activity of mismatched dsRNA. C3 cell cultures (1.5×10^6 cells per 10 ml) were incubated for 24 hr at 36°C in growth medium supplemented with 0, 0.4, 2, 10, or 50 μg of mismatched dsRNA per ml of culture. Following this incubation the cultures were challenged with HTLV-III (2.6×10^6 cpm of RT activity) as described and further incubated in 20 ml of fresh effector-free growth medium for 7 days. Cell densities were proportionately reduced, and media were replaced on days 3 and 5 of incubation.

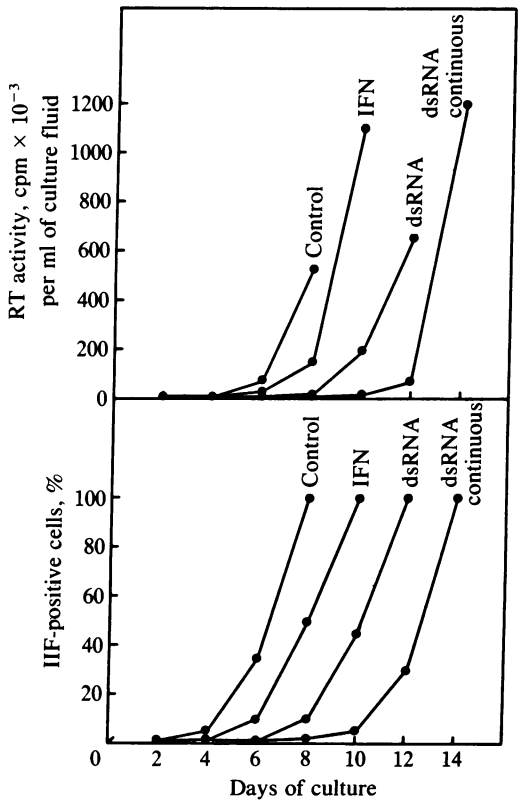

FIG. 2. Time course of HTLV-III infection in C3 cells unprotected and protected by mismatched dsRNA or IFN. C3 cell cultures (1.5×10^6 cells per 10 ml) were incubated for 24 hr at 36°C in effector-free growth medium or growth medium supplemented with mismatched dsRNA (50 μg per ml) or IFN (150 IU each of rIFN-α, IFN-β, and IFN-γ per ml). Following this incubation the cultures were challenged with HTLV-III (3.1×10^6 cpm of RT activity) as described and further incubated in 20 ml of effector-free growth medium, with the exception of a mismatched dsRNA-treated culture, which was maintained in 20 ml of growth medium supplemented with mismatched dsRNA (50 μg/ml). Samples were obtained, cell densities were proportionately reduced, and media were replaced every 2 days.

dsRNA (19.6×10^4 cpm/ml), and 12 days for continuous incubation with mismatched dsRNA (7.4×10^4 cpm/ml). Also consistent with these patterns were the time frames for all cells in culture to become productively infected with HIV as indicated by 100% of cells expressing p24 and by elevated levels of RT activity in culture fluids. These time frames were 8, 10, 12, and 14 days for the control, IFN, mismatched dsRNA and mismatched dsRNA (continuous) cultures, respectively.

Effect of Mismatched dsRNA and IFN on the Lytic Response of C3 Cells to HIV Infection. Fig. 3 compares the antiviral activities of mismatched dsRNA and IFN with respect to protection from the virus-induced lytic response in association with HIV p24 expression and virus production 7 days after virus (HTLV-III) challenge. Cell lysis was most apparent in the control (no effector) culture (67% cell reduction), which also contained the highest percent of cells expressing p24 (100%) and the greatest amount of RT activity in culture fluid (1163×10^3 cpm/ml). Pretreatment and continued incubation of cells with mismatched dsRNA (50 μg/ml) afforded the greatest protection (0% cell reduction) which was associated with a total absence of RT activity and less than 1% of cells expressing p24. Protection was also observed if cultures were exposed to mismatched dsRNA (50 μg/ml) a single time immediately prior to virus challenge where a 10% reduction in cells was observed in association with 12% of

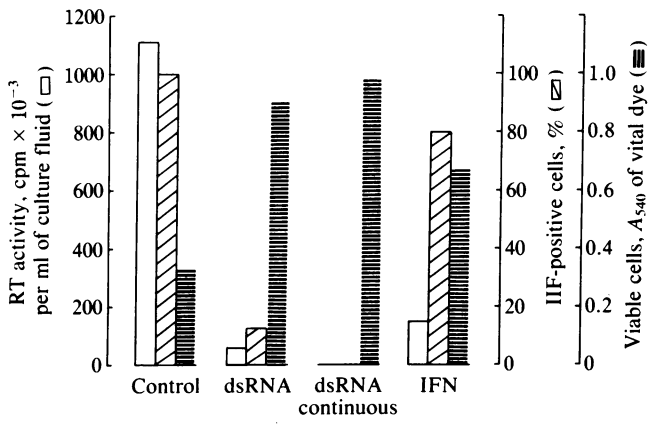

FIG. 3. Comparison of the lytic response to viral antigen expression and virus replication in mismatched dsRNA-treated and IFN-treated C3 cells subsequently challenged with HTLV-III. C3 cell cultures (1.5×10^6 cells per 10 ml) were incubated in the absence or presence of mismatched dsRNA (50 μg/ml) or IFN (150 IU each of rIFN-α, IFN-β, and IFN-γ per ml) and challenged with HTLV-III (1.7×10^6 cpm of RT activity) as described in the legend to Fig. 2. Cell densities were proportionately reduced and media was replaced every 2 days following virus challenge. Samples for IIF, RT activity, and neutral red staining were obtained on day 8.

cells expressing p24 and 63×10^3 cpm RT activity per ml of culture fluid. In contrast, the extent of lysis resistance afforded by an IFN cocktail pretreatment [150 IU of (each) rIFN-α, rIFN-β, and rIFN-γ per ml of culture] was less significant (32% reduction in cells with 70% of cells expressing p24 and culture fluid containing 155×10^3 cpm RT activity per ml).

Anti-HIV Activity of Mismatched dsRNA and Various IFNs in Cultures of CEM Cells. Fig. 4 illustrates the antiviral activity observed after 15 days in cultures of CEM cells incubated with either mismatched dsRNA (50 μg/ml), rIFN-α (250 IU/ml), IFN-β (250 IU/ml), or IFN-γ (50 IU/ml) prior to challenge with the HIV isolate LAV. Greatest antiviral activity was observed in cultures incubated with mismatched dsRNA where only 1% of cells were positive for p24 expression and little RT activity was observed in culture fluids (5×10^4 cpm per ml). This contrasted with the control culture in which 48% of cells expressed p24, while RT activity in the culture fluid was 116×10^4 cpm. Antiviral activity provided by mismatched dsRNA was greater than that observed for rIFN-α (5% of cells p24 positive, 50×10^4 cpm

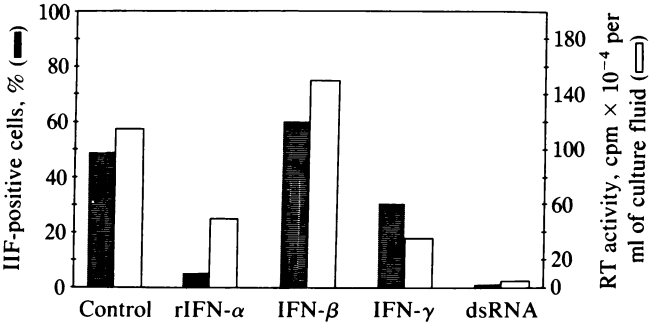

FIG. 4. Antiviral activity of mismatched dsRNA and IFN on CEM cells challenged with LAV. CEM cell cultures (1.0×10^7 cells per 10 ml) were incubated in the absence or presence of rIFN-α (250 IU/ml), IFN-β (250 IU/ml), IFN-γ (50 IU/ml), or mismatched dsRNA (50 μg/ml) for 18 hr at 36°C and challenged with LAV (0.6×10^6 cpm of RT activity). Cultures were then washed in RPMI 1640 medium and incubated in 20 ml of growth medium for 15 days with the media being replaced and cell densities being proportionately reduced on days 3, 5, 7, 9, 11, and 13.

Table 1. Effect of mismatched dsRNA on cell division, RNA and protein synthesis, and virus replication

Cell line*	Cell doublings†		[³H]Uridine, cpm per 10^6 cells		³H-labeled amino acids, cpm per 10^6 cells		RT activity, cpm per 10^6 cells per ml‡	
	−	+	−	+	−	+	−	+
CEM/HTLV-III	3.1	3.1	716	732	1,535	1,666	2,082	1,951
H9/HTLV-III	2.4	2.3	1680	1767	16,181	16,778	26,860	29,650
CEM	3.1	3.0	864	895	1,869	2,198	NT§	NT
C3	2.1	2.1	7069	7163	15,637	15,777	NT	NT

*Cultures were incubated for 4 days in the absence (−) or presence (+) of mismatched dsRNA at 50 μg/ml as described.
†Initial cell densities were: CEM/HTLV-III, 1.0×10^7 cells per culture; H9/HTLV-III, 6.2×10^6 cells per culture; CEM, 1.0×10^7 cells per culture; and C3, 2.0×10^6 cells per culture.
‡cpm obtained at 40% counting efficiency to within 2% sigma error.
§NT, not tested.

RT activity per ml) and IFN-γ (30% of cells p24 positive, 36×10^4 cpm RT activity per ml). No anti-HIV activity was observed for IFN-β in these experiments.

Cell Division, RNA and Protein Synthesis, and Virus Replication. Mismatched dsRNA at 50 μg/ml was examined for an effect on cell division, RNA and protein synthesis, and virus replication (Table 1). CEM and CEM/HTLV-III cell densities increased by 3.0–3.1 doublings during a 4-day incubation period whether in the absence or presence of mismatched dsRNA. Similarly, C3 cell densities increased by 2.1 doublings, while H9/HTLV-III cell densities increased by 2.3–2.4 doublings whether in the absence or presence of mismatched dsRNA. Incorporation of [³H]uridine and ³H-labeled amino acid was also shown to be unaffected by mismatched dsRNA in each of the above cell lines. Furthermore, mismatched dsRNA had no effect on HTLV-III replication in cultures of CEM/HTLV-III or H9/HTLV-III cells where differences in RT activity were less than the 10% average variability we commonly observe in parallel cultures.

We also examined the effects of an IFN mixture (150 IU each of IFN-α, rIFN-β, and rIFN-γ per ml) on cell division in cultures of C3, CEM, CEM/LAV, and H9/HTLV-III cells, as well as viral replication in the latter two cell lines (Table 2). Whereas IFN had no effect on cell division in C3 and CEM cells (<4% reduction in cell doubling), a mild inhibition of cell replication was observed in cultures of CEM/LAV cells (11% reduction in cell doublings), whereas a greater inhibition was observed in cultures of H9/HTLV-III cells (56% reduction in cell doubling). LAV replication in CEM/LAV cultures and HTLV-III replication in H9/HTLV-III cultures were both dramatically reduced in the presence of IFN (100% and 55% reductions, respectively).

Table 2. Effect of interferon on cell division and virus replication

Cell line*	Cell doublings†		RT activity, cpm × 10^{-3} per 10^6 cells per ml	
	−IFN	+IFN	−IFN	+IFN
C3	1.93	1.86	NT‡	NT
CEM	3.07	3.05	NT	NT
CEM/LAV	3.08	2.75	65	0
H9/HTLV-III	3.00	1.33	25,000	11,200

*Cultures were incubated for 4 days in the absence (−) or presence (+) of the combined IFNs α, recombinant β, and recombinant γ at 150 units each per ml.
†Initial cell densities were: C3, 2.1×10^6 cells per culture; CEM, 5.1×10^6 cells per culture; CEM/LAV, 5.0×10^6 cells per culture; and H9/HTLV-III, 2.3×10^6 cells per culture.
‡NT, not tested.

DISCUSSION

In this report, we have demonstrated the ability of a mismatched dsRNA, $r(I)_n \cdot r(C_{12}\text{-}U)_n$, to afford significant protection of T-cells from HIV infection *in vitro*. Antiviral activity was demonstrated in two T-cell lines, C3 and CEM, and against two HIV isolates, HTLV-III and LAV (Figs. 1–4). This antiviral activity was not attributed to an effect on cell division or RNA and protein synthesis (Table 1).

Because dsRNAs are potent inducers of the various molecular forms of IFN (13, 17), and because IFN has been shown here (Figs. 2–4) and elsewhere (15, 16) to possess anti-HIV activity *in vitro*, then IFN could at least in part be responsible for the anti-HIV activity of mismatched dsRNA. Evidence suggesting a mechanism for mismatched dsRNA distinct from IFN induction comes from our observation that mismatched dsRNA was a more potent anti-HIV agent than IFN-α, IFN-β, or IFN-γ alone, or all three IFNs combined, at doses (50–250 IU/ml) previously shown to be optimal for anti-HIV activity *in vitro* (15, 16). Further evidence comes from our observation that treatment with an IFN-α,β,γ mixture that provided less anti-HIV activity in uninfected cells than mismatched dsRNA (Figs. 2 and 3) also inhibited virus replication in cultures of CEM/LAV and H9/HTLV-III cells (Table 2), whereas mismatched dsRNA had no effect on the replication of these viruses (Table 1).

Variations in anti-HIV activities of different IFN preparations were evident from our studies which showed that IFN-γ was active while IFN-β was inactive against LAV in CEM cells (Fig. 4), whereas the opposite was true for other preparations of these IFNs in PBMCs challenged with HTLV-III, LAV, and AIDS-associated retrovirus as reported elsewhere (16). The apparent greater efficacy of mismatched dsRNA over IFN for protecting T-cells from HIV infection *in vitro* may be artifactual in the sense that mismatched dsRNA could have induced the natural IFN products of the target cells, these natural IFNs in turn being more potent than the IFN preparations added exogenously. Since dsRNA not only induces IFN biosynthesis, but activates certain IFN-induced enzymes (13, 24), the greater efficacy of mismatched dsRNA in protecting target T-cells from HIV infection may be the direct result of enzyme activity dependent on dsRNA activation. One such mediator of IFN action could be the activity of (2′-5′)oligoadenylate synthetase, an enzyme induced by IFN that synthesizes 2′–5′ oligoadenylate molecules, which in turn function to activate a latent ribonuclease specific for single-stranded RNA (13, 14). Thus, the amount of IFN induced by dsRNA, together with the IFN-mediated pathway affected, may also explain the observed difference between IFN and mismatched dsRNA on the *in vitro* replication of HIV. For example, low levels of IFN induction concomitant with activation of a specific IFN-

mediated process may be sufficient to exert antiviral activity in cells challenged with HIV, but insufficient to inhibit virus replication in cells already productively infected. Although not studied in this report, it is possible that exogenous IFNs in combination with mismatched dsRNA may result in greater inhibition of HIV production in cells already infected with HIV than with the IFNs alone.

The ability of mismatched dsRNA to protect target T-cells from HIV infection *in vitro* suggests that it may have potential efficacy in the treatment of AIDS. Since the activity of mismatched dsRNA demonstrated in this report is to protect uninfected cells, the efficacy of mismatched dsRNA as a single anti-HIV drug may be greater in ARC (AIDS related complex) patients than in end-stage AIDS. The higher levels of T-helper cells and the presence of functional natural killer cells in AIDS related complex patients suggest the presence of uninfected stem cell populations of these HIV target cells whose protection from HIV infection by mismatched dsRNA could theoretically result in the reconstitution of the abrogated immune functions associated with AIDS. In consideration of the current paucity of potential anti-HIV drugs with acceptable associated toxicities (12), and on the basis of our *in vitro* data, mismatched dsRNA appears to be a major candidate drug for the treatment of AIDS related complex and possibly AIDS. Fortunately, the severe *in vivo* toxicities associated with the dsRNAs are not observed with the mismatched analogue, Ampligen (21, 22).

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