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TITLE: TRANSDOMINANT REV AND PROTEASE MUTANT PROTEINS OF HIV-SIV AS POTENTIAL ANTIVIRAL AGENTS IN VITRO AND IN VIVO (AIDS)

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13. ABSTRACT (*Maximum 200 words*)

The major goal of this contract is to use gene therapy to target essential genes of HIV-SIV in order to inhibit virus expression. Our initial focus was to generate transdominant mutants of rev and protease genes to evaluate them in an in vivo model. During the last two years, we have expanded our approaches to include use of antisense oligodeoxynucleotides (ODN) directed to the Rev Response Element (RRE) and ribozymes that target viral mRNAs. The ribozyme approach, in particular, has yielded extremely encouraging positive data. We showed that a hairpin ribozyme designed to cleave HIV-1 RNA in the 5' leader sequence suppressed virus expression in Hela cells co-transfected with proviral DNA. Human $CD4^+$ T cell lines (Jurkat and Molt 4/8) transducted by a retroviral vector expressing ribozyme were resistant to challenge from diverse strains of HIV-1, including an uncloned clinical isolate. Transduction of primary lymphocytes with the ribozyme vector also completely blocked infection by HIV-1. In addition to inhibiting virus expression from integrated proviral DNA, expression of ribozyme also significantly decreased (by approximately

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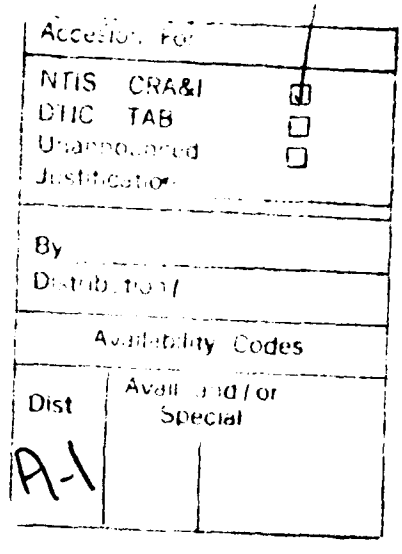
13. Abstract (continued)

100 fold) the efficiency of incoming virus to synthesize viral DNA. These results indicate that transfer and expression of the ribozyme gene interferred with both early and late events in the HIV replication cycle and conferred long-term resistance to HIV-1 infection.



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FOREWORD

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_____For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

_____In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature Jennie Wongstaal Date 10/28/93

(3) QUARTERLY REPORT

1. Contract No. DAMD17-90-C-0094 2. Report Date 10/26/93

3. Reporting period from 12/4/92 to 9/3/93

4. PI Flossie Wong-Staal, PhD 5. Telephone No. (619) 534-7958

6. Institution University of California, San Diego

7. Project Title: Transdominant Rev and Protease Mutant Proteins of HIV/SIV as Potential Antiviral Agents in Vitro and In Vivo

8. Current staff, with percent effort of each on project.

Gunter Kraus, PhD 50%	Zhen Qin Xia 50%
Michael Mamounas, PhD. 95%	Marina Heusch 25%
Flossiew Wong-Staal, PhD 16%	Various Student Lab Helpers 40%

9. Contract expenditures to date:

Personnel	98,443.53	Supplies	20,097.58
Travel	322.00	Other	4,795.95
Equipment	-0-	IDC	46,230.12
		TOTAL	169,889.18

10. Comments on administrative and logistical matters.

11. Use additional page(s), as necessary, to describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this isn't possible. Include data where possible.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

I. Introduction

I. Introduction

The life cycle of HIV provides many attractive steps for potential intervention by gene therapy, including transdominant mutant gag and envelope genes which would interfere with virus entry, TAR decoys to inhibit transcription and trans activation, RRE decoys and transdominant Rev mutants to inhibit RNA processing,to give a few examples (1). Antisense RNA or DNA and ribozymes have recently been used to target the same step in HIV-1 life cycle, namely the utilization of viral mRNA (reviewed in ref. 2). Conventional antisense RNAs and DNA have been shown to impair gene expression (3) and have potential as antiviral and anticancer agents. Ribozymes are RNA molecules that contain anti-sense sequences for specific recognition, and a RNA-cleaving enzymatic activity. The catalytic strand cleaves a specific site in a target RNA at greater than stoichiometric concentrations (4-6). The ribozyme technology has emerged as a potentially powerful extension of the antisense approach to gene inactivation (2). Intracellular expression of hammerhead ribozymes (7-9) and a hairpin ribozyme (10) directed against HIV-1 RNA has been shown to confer significant resistance to HIV-1 infection.

Hairpin ribozymes were originally described for the negative strand satellite RNA of the tobacco ringspot virus [(-)sTRSV]. This small RNA genome utilizes cis self-cleaving ribozyme in rolling-circle replication (11-17). The ribozyme activity in vitro and in vivo requires a secondary structure that contains four consensus domains. The original native minimum sequence hairpin ribozyme had 50 nt catalytic RNA cleaving a 14 nt substrate RNA in a trans reaction. The catalytic RNA/substrate RNA complex forms a type of hairpin two-dimensional structure having four helical domains and five loop structures. Two helices form between the substrate and the ribozyme which allow specificity of binding. Located between these two helices in the substrate is a N*GUC sequence with the GUC being a required sequence and cleavage occurring at the * position. Certain sequences are cleaved with high efficiency in vitro: Kcat and Km being 2.1/min and 30 nM respectively for the native sequence at pH 7.5, 37oC, low salt and 12 mM Mg2+ (13). Since cleavage occurs efficiently in vitro under near physiological conditions, the hairpin ribozyme may also be functionally favorable in vivo .

We initiated a collaboration with Dr. Arnold Hampel (University of Northern Illinois) two years ago to investigate if a hairpin ribozyme against HIV would be effective in virus inhibition. The first HIV-1 ribozyme that we have analyzed was engineered to cleave the 5'-leader sequence of HIV-1 HXB2 isolate clone message at positions +111/112 from the cap site (10). The extremely encouraging results so far obtained with this ribozyme urged us to expand

our efforts to seriously develop ribozyme gene therapy against AIDS.

Our objectives are as follows:

1. To design and construct ribozymes targeting conserved sequences of HIV-1 RNA.

2. To evaluate the capacity of the ribozymes to inhibit replication of HIV in transient transfection systems.

3. To insert ribozymes in amphotropic murine retrovirus vector and optimize expression in human T-cells using different internal promoters.

4. To transduce PBL cells or neoplastic T-cell lines with retrovirus expressing the ribozymes to determine if uninfected cells could be protected from de novo infection and if chronically infected cells would be repressed in virus production.

5. To determine if SCID-Hu mice treated with antisense oligonucleotides or reconstituted with human PBL transduced by ribozyme-expressing retrovirus vector would be refractory to HIV infection and/or virus induced CD4 cell depletion.

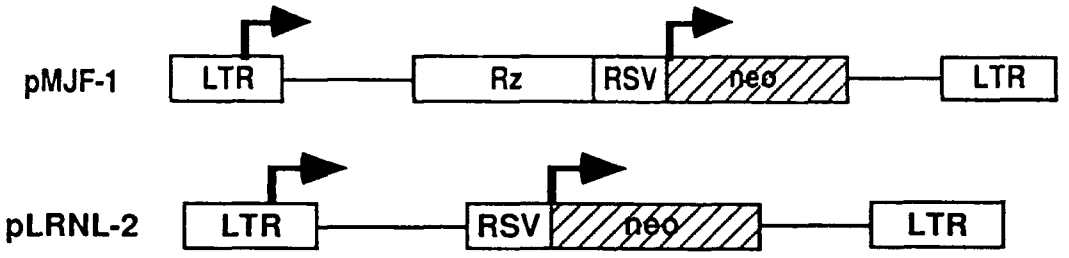
We have now accomplished all but last of the specific aims.

II. PROGRESS (BODY)

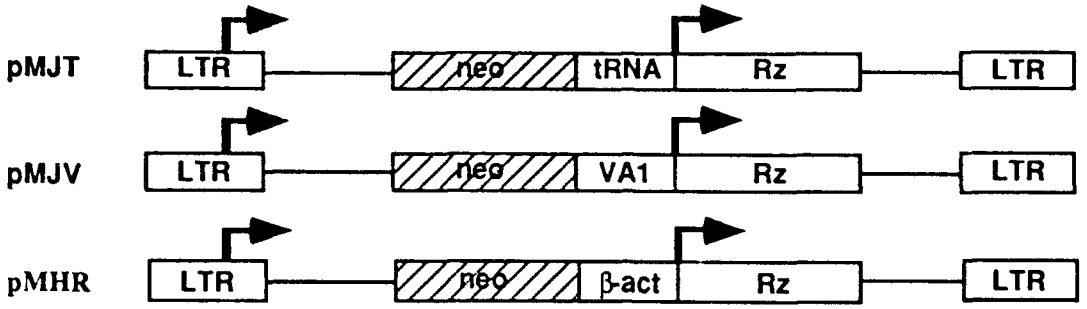
II. Progress

A. Construction of Retroviral Vectors Containing the HIV-1 Hairpin Ribozyme.

To use an efficient system to deliver ribozymes to human hematoportic cells, we have inserted the ribozyme gene into retroviral vectors. Plasmid pMJF-1 contains the ribozyme gene (Rz) inserted downstream of the 5' moloney murine leukemia virus (MoMLV)LTR. To allow selection of the transduced cells, a RSV-neo cassette containing the bacterial neomycin phosphotransferase (neo) gene controlled by the promoter of Rous Sarcoma Virus (RSV) was inserted downstream of the ribozyme gene. The arrows indicate the position of transcription initiation and the direction of transcription. pLRNL-2 is a control vector without ribozyme.



We were also exploring the retroviral vector system by combining the conventional MoMLV-based vectors with pol III expression control units. We have constructed three vectors which contain the neo gene controlled by the 5'LTR and the ribozyme controlled by an internal promoter. pMJT and pMJV express the ribozyme gene from the human tRNAval promoter and adenovirus VA1 promoter, respectively. We also constructed pMHR which expresses the ribozyme gene from the human b-actin promoter (pol II).



B. The HIV-1 Leader Sequence Ribozyme Inhibits Diverse HIV-1 Strains.

A necessary feature of any effective therapeutic agent against AIDS will be its ability to inhibit different HIV-1 strains. In addition to the results we obtained for strains HXBII and SF-2 (18), we also tested strains Eli and MN which exhibit varying genetic distance from HXB2, with Eli, a Zairean strain, being the most distant. The leader sequence targeted by the ribozyme under study is completely conserved among known HIV-1 strains, with the exception of that of MN, which shows one base mismatch (from G-C to A-C). Such a mismatch at position +6 relative to the CUG cleavage site has been shown to be tolerated to some extent in in vitro cleavage reactions (19). Whether it would also be tolerated in vivo was not known.

Alignment of HIV-1 Leader Sequence
(The Hairpin Ribozyme Target)

HXB2	TG CCC GTC TGT TGT GT
MN	TG CCC GTC TGT TaT GT
SF2	TG CCC GTC TGT TGT GT
RF	TG CCC GTC TGT TGT GT
HAN	TG CCC GTC TGT TGT GT
ELI	TG CCC GTC TGT TGT GT
Z2Z6	TG CCC GTC TGT TGT GT

It is possible that differences in surrounding sequences can affect accessibility and ribozyme cleavage. We addressed this important diversity issue by using the less time-consuming transient transfection assay with the retroviral vectors that we constructed. We co-transfected the HIV-1 DNA with retroviral vectors carrying the ribozyme gene driven by internal promoters. Ribozyme gene expression was detected by RNA dot blot in cells transfected by the ribozyme containing DNAs, but not in control cells transfected with vector alone (18). Expression of p24 gag in culture medium was then determined 48 hours after transfection. As shown in Fig.2, co-transfection of the control vector DNA pLRNL-2 together with Eli and MN resulted in high levels of p24 expression. As seen in Fig. 1A, all three retroviral vectors carrying the ribozyme gene showed significant inhibition of Eli expression. Slightly less but still significant inhibition was observed for the MN strain (Fig. 1B), suggesting that the single base substitution in the target sequence does have some effect on ribozyme activity. More importantly, this result indicates that the hairpin ribozyme can tolerate certain conservative base changes, which is an important feature of the hairpin ribozyme to be used as the anti-HIV reagent. In fact, we observed greater than 95% inhibition of MN in the ribozyme transduced Jurkat cell line by two different retroviral vectors up to 4 weeks (see next section).

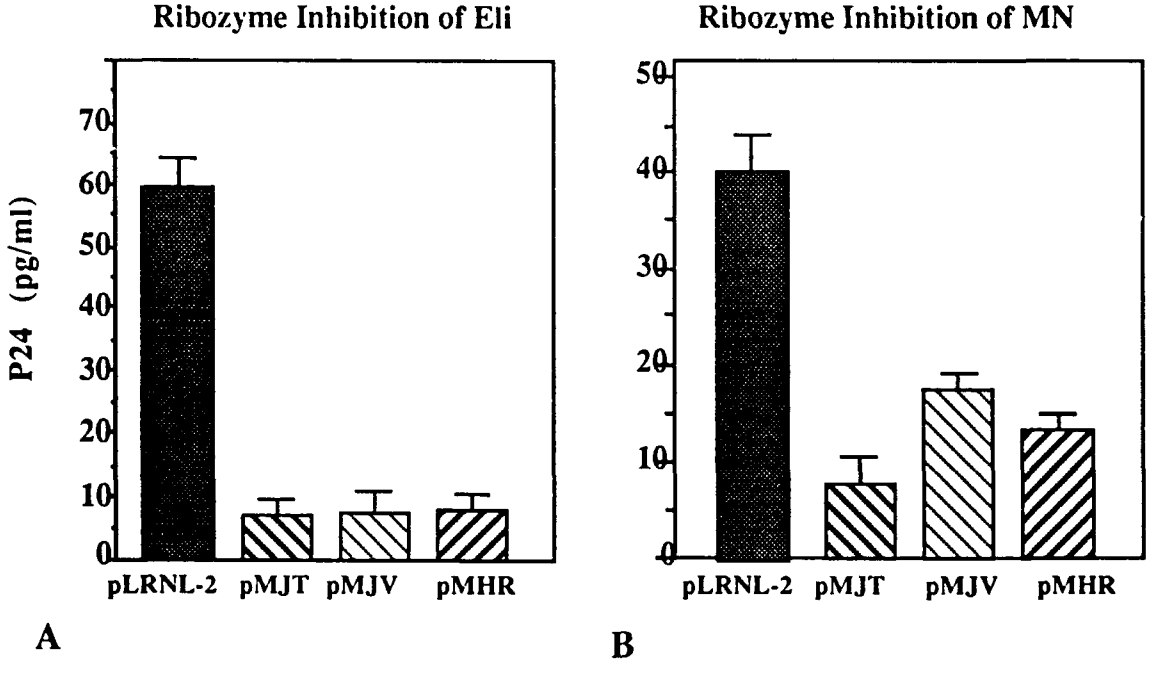


Figure 1. Effect of the ribozyme on HIV-1 expression in a transient assay. pMJT, pMJV, and pMHR are retroviral vectors carrying the ribozyme driven by the internal tRNA, VA1 and b-actin promoter respectively. pLRNL-2 is a retroviral vector without the ribozyme gene.

C. Retrovirus-mediated gene transfer of the anti-HIV-1 ribozyme gene into human T cell lines.

To test whether transduction of human CD4+ T cell lines by the ribozyme-containing retroviral vectors can confer resistance to HIV-1 replication, we transfected pMJF-1, pMJT or a control plasmid pLNL6 (20) containing the neo gene inserted between the two LTRs of MoMLV into the amphotropic packaging cells PA317. Infectious virus was harvested two days after transfection and used to infect Jurkat cells. Neo-resistant (neoR) cells were then pooled and challenged with HIV-1 at a multiplicity of infection of 0.01. Up to thirty-five days after infection with the HXB2 strain of HIV-1, less than 1% of HIV reverse transcriptase (RT) was detected in the medium of MJF-1 virus-infected cells when compared with the pick RT activity in the medium of LNL6 virus-infected cells (Fig. 2, Detection of RT activity for the control was terminated on day 19 due to extensive cell death).

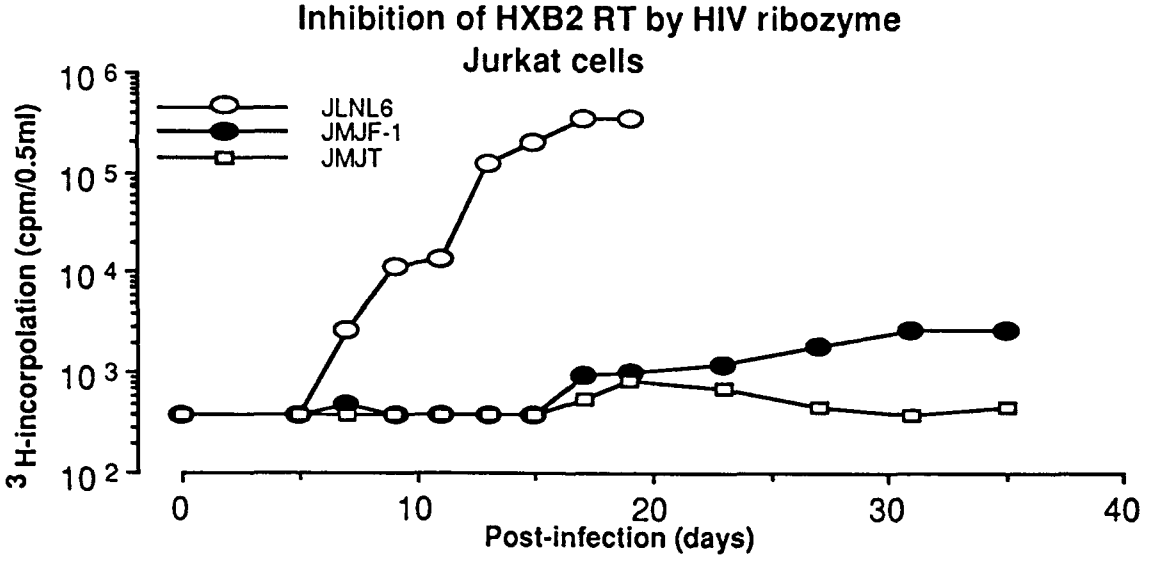


Figure 2. Inhibition of HIV-1 HXB2 strain RT activity by the HIV ribozyme in Jurkat cells. $3X10^5$ Jurkat cells (MLNL-6, MMJF-1, or MMJT) transduced by retroviral vector, pLNL-6, pMJF-1 or pMJT, were infected with HXB2 virus. The cells were divided every 2 days to the starting cell number ($3X10^5$) and the reverse transcriptase (RT) activity in the supernatant was determined.

Moreover, no RT activity (below background level: 10^3 cpm) can be detected in the medium of MJT virus-infected cell up to 35 days, suggesting that HXB2 expression is completely blocked in this cell line (The experiment was terminated at

day 35). Similar results with less extensive inhibition were obtained when the MN strain of HIV-1 was used to infect the pooled neor Jurkat cells (not shown). Thus, this hairpin ribozyme which specifically cleaves a conserved region among different strains of HIV-1 can efficiently inhibit HIV-1 replication in Jurkat cells when delivered via retroviral vectors. We used RNA PCR to detect neo as well as the ribozyme gene products. Neo gene product was detected in all the stable cell lines containing retroviral vectors, including pLNL-6. The ribozyme gene product could only be detected in the stable cell lines containing retroviral vectors with the ribozyme gene (data not shown).

Infectivity of the supernatants collected on days 17 and 35 was determined using MT-2 cells (HTLV-I carring human CD4+ T cell line) and the amount of p24 Ag in the same supernatants were measured by antigen capture assays. No infectivity was detected in the supernatant of JMJT on both day 17 and day 35 whereas low infectivity ($10^{1.25}$ $TCID_{50}$/ml) was detected in JMJF-1 on day 35, which was three logs lower than the TCID50/ml of the supernatant from JLNL-6 cells (Table 1). Low p24 antigen levels were detectable in JMJT supernatants on days 17 and 35 (Table 1). In MN infected JMJT and JMJF-1, the RT activity had been suppressed to less than 2% and 5%, respectively, up to 27 days after infection when compared with the peak value in MN infected JLNL6 (not shown). Infectious virus was also recoverable on days 13 and 23 (Table 1). These results demonstrate a greater than four logs reduction in recovered virus titer from MJT transduced Jurkat cells when HXB-2 was used as the challenge virus. Even with MN, which is the single exception haveing one mismatched nucleotide in the target sequence, we still observed close to 3 logs reduction at day 23. HXB-2 and MN viruses recovered from JMJF-1 were equally susceptible to ribozyme inhibition in rechallenge experiments (data not shown but presented in Yamada et al. in Appendix)

Table 1.

Reduction of Virus Titer and Antigen in Ribozyme Transduced Jurkat cells After Virus Challenge

Cell line	Virus strain	DPI	TCID50/ml	p24 (pg/ml)
JLNL6	HXB2	17	104.25	813000
JMJF-1		0	-	<15
		17	Not detectable	887
		35	101.25	1462
JMJT		0	-	<15
		17	Not detectable	163
		35	Not detectable	104
JLNL6	MN	13	104.00	731000
JMJF-1		0	-	<15
		13	101.25	1810
		23	102.75	-
JMJT		0	-	<15
		13	Not detectable	948

Infectious titer (TCID50) in the culture supernatants of JLNL6, JMJF-1 and JMJT cells infected with HIV-1 HXB2 or MN strains on the day indicated were determined using MT-2 cells as described previously (17). The level of extracellular p24 antigen was determined by the antigen capture ELISA test (Coulter) - = Not Done.

D. The Ribozyme Also Inhibits Early Events in Virus Replication

Our previous observation that transient co-transfection of viral DNA and ribozyme gene led to reduction of virus RNA and protein production (13, 18) demonstrated that the ribozyme inhibited virus gene expression, presumably by cleavage of the transcribed viral mRNA. However, since the viral genomic RNA also contained the target sequence, the ribozyme can potentially cleave the incoming viral RNA and prevent the establishment of infection as well. To examine if the ribozyme indeed interfered with early events of virus replication, we measured proviral DNA syntheses 6 or 18 hours after infection with HIV-1 in cells with and without expression of ribozyme, using a semi-quantitative, nested double PCR procedure. As an internal control, a primer pair for b-globin gene was added to the reaction tubes after the 10 th cycle of the first PCR to avoid potential interference

of b-globin product with the amplification of LTR sequences, since the amont of b-globinDNA was estimated to be higher than that of HIV-1 DNA in the cellular extracts due to low percentage of infected cells. To estimate the relative amounts of HIV-1 DNA in the cellular DNA extracts, different concentraions (0.05fg to 500fg/ml) of HIV-1 SF2 -containing plasmid DNA was amplified using the same primers and conditions. These experiments demonstrated that JMJT contained approximately 50 to 100 times less HIV-1 DNA than JLNL6 at the same input of cellular DNA (Data not shown). These result showed that the ribozyme also efficiently interfered with early events in viral replication, presumably by cleaving incoming viral RNA.

E. Transduction of Human Peripheral Blood Lymphocytes (PBL) with retroviral vectors.

As a further step towards the retroviral vector-mediated HIV ribozyme gene therapy, we recently investigated the feasibility of using retroviral vectors to transduce human peripheral blood lymphocytes (PBL). This is not only of significance for studying the efficacy of the ribozyme in human primary T cells; it is also important for the reason that PBL system is ideal for studying the ribozyme effect on the replication of HIV clinical isolates. Furthermore, it is the first step for testing HIV ribozymes gene therapy using hu-PBL-SCID mice model. Under stimulatory conditions, lymphocytes proliferate and can be transduced by retroviral viral vector encoding the neomycin resistance gene (21). Non-transduced PBMCs can then be eliminated by culture in the presence of the antibiotic G418. It is possible for the resistant recombinants to be expanded as much as 1000 times if maintained in IL-2 supplemented media (22). We have successfully transduced human PBMCs (without depletion of macrophages from peripheral blood lymphocytes) with the retroviral vectors pMJT, pMJF-1 and pLNL-6 using the following conditions. Ficoll-hypaque purified PBMCs were resuspended in RPMI + 10% FCS + PHA-P for 3 days. After activation, lymphocytes were continuously maintained in IL-2 (20U/ml)-supplemented medium. Stimulated cultures were either incubated with supernatants or co-cultivated with PA317 cell lines producing the different retroviral vectors. Following three days of transduction, recombinants were selected in G418 (400mg/ml) IL-2 (20U/ml)-supplemented medium for 9-10 days. From 1x106 initial PBMCs we generated approximately $2x10^6$ G418 resistant recombinants. As shown in fig. 3, PBL transduced with the pMJT vector also resisted infection by HIV-1/HXB-2 over a period of $3^1/_2$ weeks in culture.

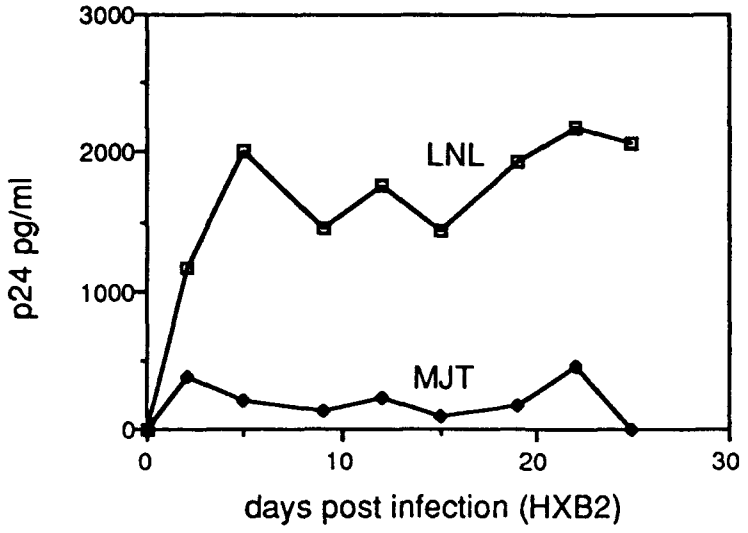


Fig. 3. Primary PBL transduced with MJT and LNL vectors and challenged with HXB-2. See text for details.

III. CONCLUSIONS

III. Conclusions

As indicated in the second annual report, we had adopted a novel therapeutic approach against HIV infection, namely the use of a hairpin ribozyme. In this report, we present encouraging data on utilizing this approaches in inhibiting HIV replication. We showed that a hairpin ribozyme designed to cleave HIV-1 RNA in the 5' leader sequence suppressed virus expression in Hela cells co-transfected with proviral DNA from diverse HIV-1 strains. We then "immunized" human $CD4^+$ T cell lines (Jurkat and Molt 4/8) intracellularly wtih the ribozyme gene delivered in murine retroviral vectors driven either by an internal human $tRNA^{val}$ (pol III)promoter or directly by the retroviral LTR. There was no apparent deleterious effect of ribozyme expression on cell proliferation or long term viability. Higher levels of ribozyme expression was consistently obtained with the pol III promoter. Cells expressing ribozyme were resistant to challenge from diverse strains of HIV-1, including an uncloned clinical isolate. No reverse transcriptase activity or virus infectivity was detectable in the culture supernatants of Jurkat cells expressing the ribozyme driven by $tRNA^{val}$ promotor up to 35 days after challenge with HIV-1/HXB-2. Transduction of primary lymphocytes with the ribozyme vector also completely blocked infection by HIV-1. In addition to inhibiting virus expression from integrated proviral DNA, expression of ribozyme also significantly decreased (by approximately 100 fold) the efficiency of incoming virus to synthesize viral DNA. These results indicate that transfer and expression of the ribozyme gene interfered with both early and late events in the HIV replication cycle and conferred long term resistance to HIV-1 infection.

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