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

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CONTRACTING ORGANIZATION: University of California, San Diego
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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 and to evaluate them in an in vivo model. Subsequent efforts have led us to develop a ribozyme gene therapy approach that seems promising. The first HIV-1 ribozyme that we have analyzed was engineered to cleave the 5'-leader sequence of HIV-1 HXB2 clone RNA at position +111/112 from the cap site. We have compiled data which established that this ribozyme, when delivered by a murine retroviral vector and expressed stably in Jurkat cells or PBL, is able to confer resistance to infection by diverse strains of HIV-1, including uncloned clinical isolates.

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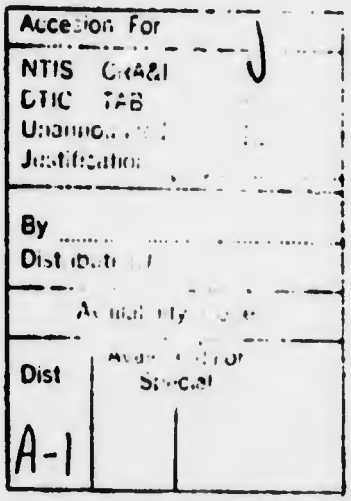
13. Abstract (continued)

Based on these positive data, we have developed a clinical protocol for a Phase 1 trial with this ribozyme vector and have obtained approval from the NIH Recombinant DNA Advisory Committee.



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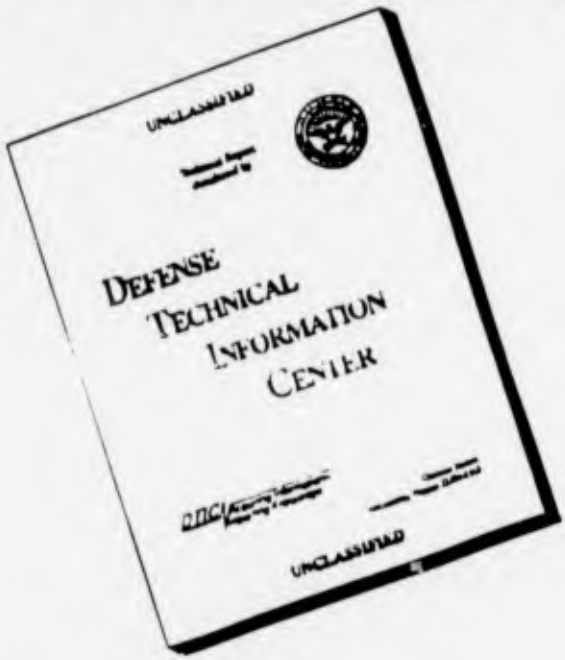

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_____For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

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

PI Signature _____ Date 4/8/94

QUARTERLY REPORT

1. Contract No. DAHD17-90-C-0094 2. Report Date 3/17/94
3. Reporting period from 9/4/93 to 2/3/94
4. PI FLOSSIE WONG-STAAL, PH.D 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 & IN VIVO
8. Current staff, with percent effort of each on project.

GUNTER KRAUS, PhD 100%
FLOSSIE WONG-STAAL 32.9%
ZHEN QIN XIA 50%
MARINA HEUSCH 50%
VARIOUS STUDENT LAB HELPERS 4C%

9. Contract expenditures to date:

Personnel	37,212.15	Supplies	4848.65
Travel	0	Other	0
Equipment	0	IDC	20272.34
		TOTAL	62333.14

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

INTRODUCTION

AIDS has become a major threat to public health on a global scale. Preventing further spread of this disease would depend on a thorough understanding of the pathogenic mechanism of these viruses and in the development of effective therapeutic agents and vaccines. The general consensus that clinical progression in HIV-infected individuals appears to critically depend on continued virus expression and recruitment of new infected cells provides the rationale for developing anti-HIV agents as therapy. Indeed, nucleoside analogs which inhibit the viral reverse transcriptase have shown clinical efficacy. However, the inherent toxicity of these drugs and the inevitable emergence of drug resistant HIV mutants in the course of treatment necessitate the continued search for novel therapeutic regimens. Gene therapy is an exciting new modality for treatment of human diseases. In addition to correction of genetic defects by insertion of functional cellular genes, gene therapy can also aim to stimulate immune responses against tumor or viral infected cells, or to inhibit expression/function of infectious, pathogenic agents.

The life cycle of HIV provides many potential intervention strategies for gene therapy: transdominant mutant viral structural proteins, TAR decoys to inhibit transcription and trans activation, RRE decoys and transdominant Rev mutants to inhibit RNA processing (see ref. 1 for review). 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 (1). Our initial goal was to develop transdominant protease and Rev proteins to inhibit HIV replication. However, we subsequently found that the ribozyme approach was more promising. Ribozymes are RNA molecules that contain anti-sense sequences for specific recognition, and a RNA-cleaving enzymatic activity. Intracellular expression of hammerhead ribozymes (2-4) and a hairpin ribozyme (5-7) 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]. The original native minimum catalytic core of the hairpin ribozyme encompassed 50 nt, cleaving a 14 nt substrate RNA in a *trans* reaction. 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, 37°C, low salt and 12 mM Mg^{2+} (8). 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) to investigate if a hairpin ribozyme against HIV would be effective in virus inhibition, and the results established that this ribozyme, when delivered by a murine retroviral vector and expressed stably in Jurkat cells or PBL, is able to confer resistance to infection by diverse strains of HIV-1, including uncloned clinical isolates.

Our objectives for the entire contract were as follows:

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

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

3. To insert the therapeutic genes 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 therapeutic genes to determine if uninfected cells could be protected from de novo infection.

5. To determine if SCID-Hu mice reconstituted with human PBL transduced by retrovirus vectors expressing the therapeutic genes would be refractory to HIV infection and/or virus induced CD4 cell depletion.

We were able to complete specific aims 1 - 4 using the hairpin ribozyme gene within the funding period.

II. Scientific Progress

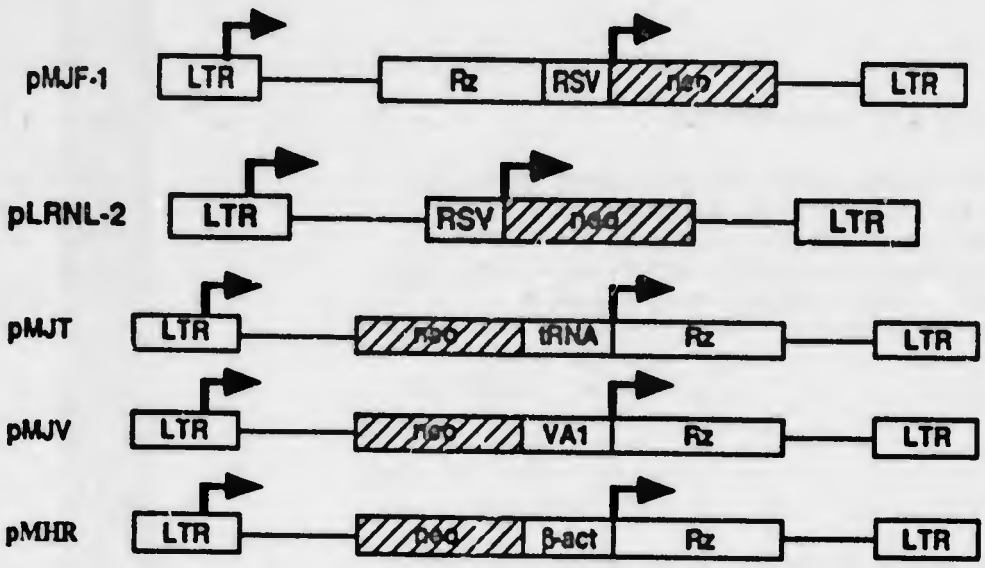
SCIENTIFIC PROGRESS

A. Transdominant Protease and Rev Mutants

We have made different mutations into the protease gene of HIV-1, but these mutants either did not express stable protease protein or expressed inactive protease that was recessive. We have also created a number mutations into HIV-1 rev cDNA. One of these mutants, p1.3, exhibited a transdominant phenotype. This mutant gene was inserted into a retrovirus vector. However, since other groups have progressed much further with the rev transdominant approach, and since we felt that expressing a viral protein intracellular would be likely to induce an immunologic response in the host, we changed our strategy to focus on the development of a ribozyme that targets the HIV-1 genome.

B. Construction of Retroviral Vectors Containing the HIV-1 Hairpin Ribozyme and Proof of Efficacy in T-cell lines.

We reported previously a hairpin ribozyme which cleaves the 5'-leader sequence of HIV-1 mRNA at the position +111/112 from the cap site (5). Expression of this ribozyme under the control of the β-actin promoter inhibits HIV-1 (HXB2) expression significantly (5), as determined by virus RNA or protein (RT, p24, Tat) levels. We further showed that the inhibitory effect of the ribozyme was specific for its target sequence, and could be attributed primarily to the catalytic, rather than the anti-sense property of the ribozyme (5). To use an efficient system to deliver ribozymes to human hematopoietic 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 expresses the ribozyme gene from the human $tRNA^{val}$ promoter in the opposite orientation of the LTR as shown, and pMJV utilizes the adenovirus VA1 promoter. We also constructed pMHR which expresses the ribozyme gene from the human b-actin promoter (pol II).



We then "immunized" human CD4+ T cell lines (Jurkat and Molt 4/8) intracellularly with 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 (7). 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.

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

As a further step towards the development of retroviral vector-mediated HIV ribozyme gene therapy, we recently investigated the feasibility of using retroviral vectors to transduce primary human peripheral blood lymphocytes (PBL). Under stimulatory conditions, lymphocytes proliferate and can be transduced by

retroviral viral vector encoding the neomycin resistance gene (8). 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 (8). We have successfully transduced human PBMCs (without depletion of macrophages from peripheral blood lymphocytes) with the retroviral vectors pMJT 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) and IL-2 (20U/ml)-supplemented medium for 9-10 days. From $1x10^6$ initial PBMCs we generated approximately $2x10^6$ G418 resistant recombinants. As shown in fig. 1a, PBL transduced with the pMJT vector also resisted infection by HIV-1/HXB-2 over a period of $3^1/_2$ weeks in culture. Similar results have been obtained with PBL from three different donors.

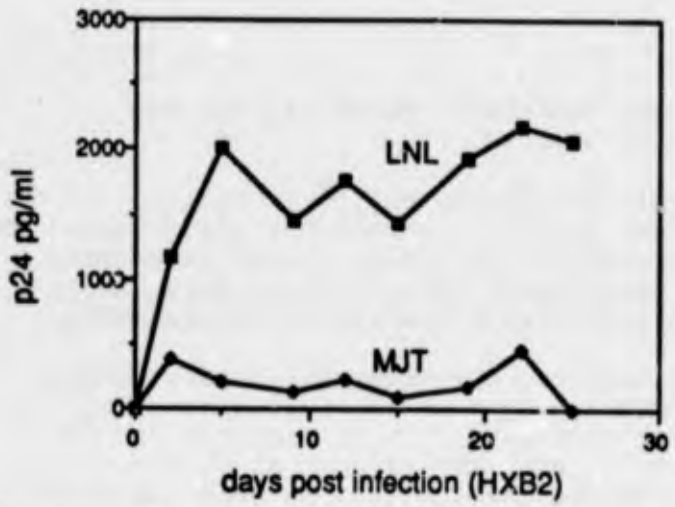


Fig. 1. Primary PBL transduced with MJT and LNL vectors and challenged with HXB-2. See text for details.

D. Transduction of CD34+ cells

M. Yu et al., in preparation

We initiated the stem cell transduction experiments using the MJ and MJV vectors. 10^6 purified CD34+ cells (9) were infected separately with cell-free recombinant virus MJT, MJV produced from cloned PA317 packaging cell lines. We used an M.O.I. of approximately 1.0 to transduce the cells in the presence of 4 mg/ml polybrene. The transductions were carried out with or without different growth factors. Subsequently, CFU assays were performed in the presence of standard growth factors without G418. We then picked 20 colonies each and performed both DNA (neo,10 colonies) and RNA PCR (Rz,10 colonies) to assess the transduction efficiency and ribozyme gene expression. High efficiency of transduction (80-100%) was obtained with both MJT and MJV vectors in the presence of growth factors (SCF or SCF + IL3/IL6). Surprisingly, even in the absence of exogenous growth factors, significant transduction (60%) was also observed (fig. 2). Although it was observed earlier that neo gene expression by the LTR was turned off shortly in hematopoetic stem cells and embryonic cells, this was not the case for the ribozyme gene, probably because it is driven by the internal promoter tRNA or VA1, the former from a house-keeping gene and the latter also from a strong pol 1II viral gene. Ninety percent of the colonies expressed detectable levels of the ribozyme gene (fig. 2).

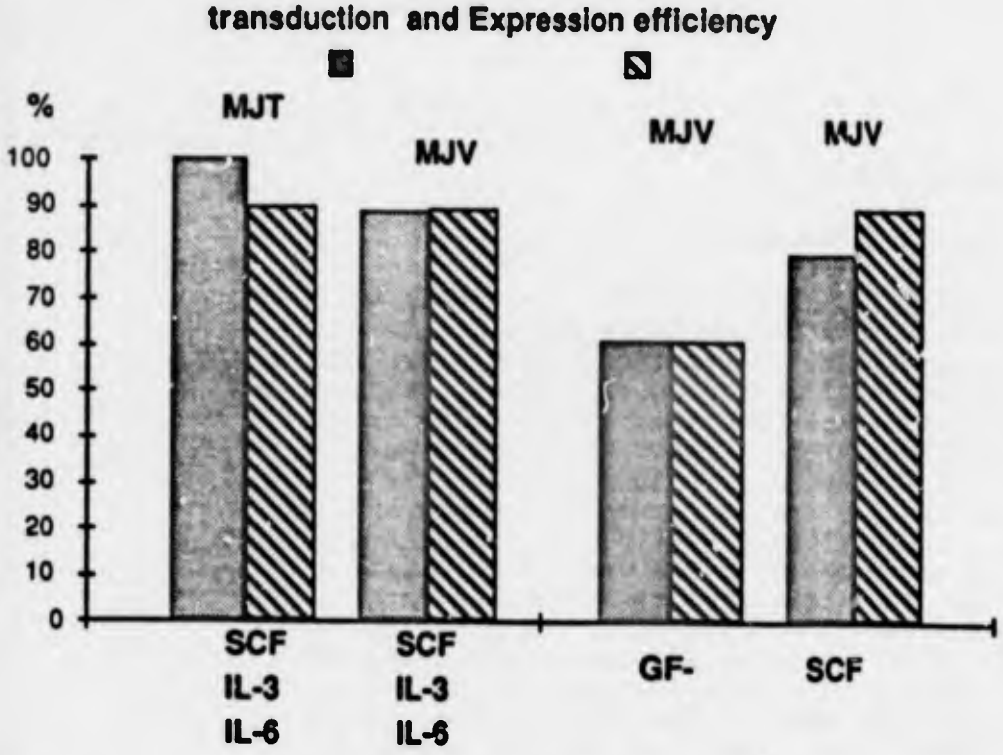
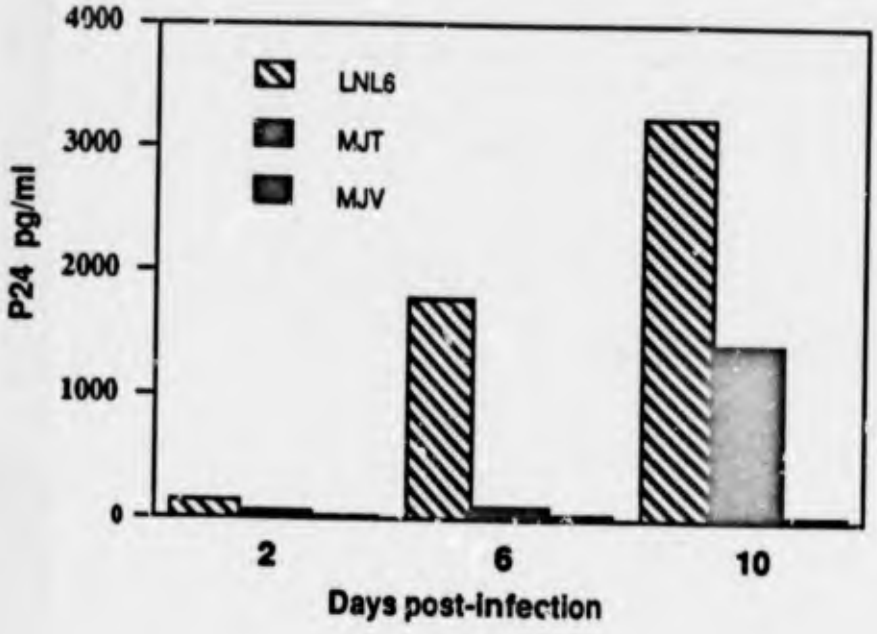


Fig. 2. Efficiency of transduction and ribozyme expression in ribozyme transduced CD34+ cells.

The efficacy of the protective genes was assessed by in vitro HIV-1 challenge. The transduced stem cells was maintained in long term culture medium (Terry Fox Lab., Vancouver, Canada) in the presence of SCF, IL3 and IL6 and GM-CSF. Under such growth conditions, most of the cells became macrophage/monocytes in 10 to 14 days. Standard HIV challenge was performed using the macrophage tropic strain HIV-1/Bal. LNL6 transduced cells was used as control. Cell culture supernatent was collected every other day and the standard HIV p24 ELISA was carried out to determine virus expression and the protective effect of the ribozyme gene product. As shown in fig. 3, transduction by the ribozyme gene, in particular, using the MJV vector, conferred resistance to the CD34+ cell derived macrophages and monocytes.

Inhibition of HIV-1 Replication in Stem Cells



III. CONCLUSIONS

CONCLUSIONS:

Twelve years into the AIDS epidemic, satisfactory treatment for this disease is still elusive. Our current understanding of AIDS pathogenesis calls for early intervention with antiviral agents. Although still in its infancy, human gene therapy holds considerable promise for the long term treatment of genetic disorders, cancer and chronic infectious diseases, including AIDS. We have explored the use of different viral inhibitory genes for "intracellular immunization" of HIV target cells. The ultimate goal is to completely reconstitute the immune system with genetically altered cells that would resist productive HIV infection. We initially worked with transdominant protease and rev mutants, but subsequently focused on developing a hairpin ribozyme in gene delivery. We demonstrated that a hairpin ribozyme targeting the 5'-leader sequence of HIV-1, when expressed stably in Jurkat cells under the control of the pol III tRNA promoter, is able to confer long term resistance to infection by diverse strains of HIV-1, including an uncloned clinical isolate. Transduction of primary lymphocytes with the ribozyme gene also confers long term resistance to infection. Finally, transduction of CD34+ stem/progenitor cells yielded high efficiency of gene transfer, and the monocyte/macrophage progeny of the transduced progenitor cells were resistant to infection by monocyte-tropic HIV.
These results suggest that ribozyme gene therapy may be feasible in the treatment of HIV infection.

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