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13. ABSTRACT (Maximum 200) Since 1992, SRA Technologies, Inc. has provided services in support of DoD interventional trials for HIV. Three working groups were established in the areas of molecular biology, cellular phenotyping and drug testing. Production level and repository services were provided for virus isolation, expansion, titration, sequencing, mutational analysis and drug susceptibility testing. Significant progress was made in the development of new assays for genotypic and phenotypic drug resistance, antibody neutralization, mutational analysis and for efficacy analysis of antiviral gene constructs. The contract contributed to discoveries in the area of drug resistance, and antibody neutralization of HIV and the development of diagnostic sequencing to support patient management in DoD clinics. The contractor was a major contributor in the discovery process leading to identification of HIV soluble and cell-associated inhibitory factors produced by CD3/CD28 antibody-stimulated cells in vitro.					
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FOREWORD

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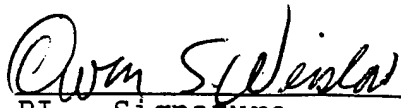
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PI - Signature

10/14/87
Date

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INTRODUCTION

Contract DAMD17-92-C-2504, Support of HIV interventional trials, was initiated in October of 1992 and was administered by Dr. Owen S. Weislow of SRA Technologies, Inc. This contract was intended to provide both research and development support of clinical studies in HIV antiviral chemotherapy, immunotherapy and immunoprophylaxis. The primary objectives of the contract, as stipulated in the contract award, are a) to develop assays to evaluate the viral and immune responses to anti-retroviral therapy including neutralization assays and drug susceptibility assays using clinical HIV isolates, b) to develop and validate assays that predict or demonstrate disease progression for use in interventional trials with an emphasis on molecular biologic approaches to viral characterization and quantification of viral burden, and c) to improve the accuracy, reliability, and cost effectiveness of clinical laboratory tests for all stages of HIV-1 and other retroviral infections. The contractor, in consultation with the contract office's representative, incorporated three working groups to develop and optimize the necessary assays in support of these clinical studies and to establish production-level protocols. The three groups include the molecular biology working group, the cellular phenotype working group and the antiviral drug testing group. The work scopes of each are briefly described below with a detailed discussion of the progress made by each during the three base years and the two option years of the contract. A Third section, the data group section, consisting of computer personnel from SRA, has been established to support the efforts of SRA to improve delivery of all necessary data elements to WRAIR personnel as stipulated in the contract award.

This is the final report describing progress made on contract DAMD17-92-C-2504 during the three base years and two option years from Oct. 1, 1992 thru Sept. 30, 1997. During this five year period a number of assays were developed or validated and moved into production status in support of ongoing clinical trials or as part of WRAIR's continuing efforts to improve patient management. Included in this arena are the molecular biology working group assays for the aa215, aa74 and aa184 mutations of reverse transcriptase (brought online in FY94-FY96), sequencing of RT and viral protease genes for monitoring drug resistance and development of the capability to provide large-scale support for analysis of HIV viral burden. The latter capability was finally applied in support of WRAIR's HIV diagnostic and other collaborative efforts. The molecular biology working group also applied these technologies to the support of RV43, RV79 and the GART (genotyping for antiretroviral drug therapy) protocol in collaboration with the NIH.

During the contracts three base years the cellular phenotyping group participated in the development and evaluation of a new infectivity reduction assay employed in virus neutralization studies and studied the neutralization kinetics of laboratory and field isolates of HIV with an eye toward improving existing assays. Particularly noteworthy are the studies performed by the cellular phenotype working group in

support of WRAIR and the Naval Medical Research Institute's, Cellular Immunology Laboratory, evaluation of long term CD4+ cell cultures for *ex-vivo* use in investigations of genetic therapies and autologous transfusions of HIV-infected patients (RV100 clinical trial). SRA's laboratories were instrumental in demonstrating the resistance of CD4+ T-cells, stimulated with solid-phase, anchored CD3 and CD28 antibodies to HIV infection. We demonstrated this resistance to be active in nature, the result of soluble factors synthesized by CD4+ cells and derived from supernatants of these cells, that inhibit infection of cellular targets normally sensitive to HIV. During the contract's option years, we determined that these supernatants possess inhibitory activity directed against both SI and NSI isolates and that factors contained in these supernatants potentiate the activity of antiviral drugs. These studies are detailed in the progress report below. Finally, the cellular phenotyping group also scaled up studies of SI/NSI isolates, performed evaluations of antiviral gene constructs and produced numerous large-scale expansions and titrated viral stocks for use by WRAIR investigators.

The antiviral drug testing group continues to evaluate clinical isolates for drug sensitivity as part of WRAIR's numerous collaborations and has, at the same time, evaluated many new putative antiviral compounds emanating from WRAIR's own laboratories and those of collaborators. Assays to accomodate preclinical evaluations of drug combinations were brought online and new, multiple drug resistant isolates have been identified, expanded, titrated and characterized, genotypically as well as phenotypically, as part of these efforts. Finally, the antiviral drug testing group has collaborated in the development of a new rapid screening system for drug resistance that could reduce the time to phenotype patients undergoing antiviral drug treatment. These activities are documented in the sections that follow.

During the option and final two years of the contract there was a significant reduction in available funding. This change was accompanied by reductions in staffing resulting in significant alterations in the contract's focus and throughput. Our concentrations in gene therapy, phenotypic evaluation of drug susceptibility and drug testing were eliminated. The cellular phenotyping working group's support of Dr. Carl June's autologous transfusion studies were similarly reduced to accomodate these staff reductions and changing workscope. The progress report which follows reflects these modifications. In it the PI reviews work previously communicated in the three base year final report and presents the studies of the two option years as modifications and extensions to both the molecular biology working group and the CD3/CD28 studies previously communicated in support of Dr. June.

PROGRESS REPORT

1. Molecular Biology Working Group

During the first two years of this contract we improved and validated the PCR assay employed in testing for the presence of AZT-resistance associated mutations at HIV-1 reverse transcriptase amino acid's 215 position. In addition, we began application of this technology to a new clinical protocol (244) sponsored by the AIDS Clinical Trials Group (ACTG), NIAID and WRAIR. We have also begun efforts to develop a similar mutational assay for the 74 mutation that confers DDI resistance. Finally, we have made improvements in our diagnostic DNA sequencing protocols for use in support of drug-resistance monitoring in clinical trials and during the contract period brought on board, at SRA's expense, a new Perkin-Elmer 377 automated sequencer to accommodate any potential increase in WRAIR's sequencing requirements. Information on the rationale behind these protocols and other background information is provided below, followed by copies of current protocols for each and a summary of work performed during the five contract years under review here.

Amplification Refractory Mutation System (ARMS)

Of great importance, in WRAIR clinical trials supported by SRA Technologies, is the monitoring of the acquisition of drug resistance. At the time this work was initiated, AZT (Zidovudine) was the primary drug used in clinical trials. While potentially providing some benefit to the patient, the usefulness of AZT and other nucleoside analogs as well as non-nucleoside RT inhibitors is limited by the tendency for almost all recipients to develop some level of resistance to the drug during the course of therapy. In order to better assess the effectiveness of treatment modalities, it is useful to have a rapid screening assay for patients that will indicate the onset of genotypic mutations associated with AZT resistance. During the course of this contract the 215 ARMS PCR assay was improved and applied to a nationwide clinical protocol for prospective analysis of the 215 mutation in clinical practice.

The theoretical basis for this assay was included in previous reports, but is described briefly here. A combination of ASO (Allele Specific Oligonucleotide) techniques and PCR has been developed that makes use of the best aspects of both techniques. Various names have been given to this technique, including the Amplification Mutation Refractory System (ARMS) or PCR Amplification of Specific Alleles (PASA), this technique takes advantage of the inability of synthetic oligonucleotide primers that are incompletely hybridized to a template to serve as effective PCR primers¹. First described by Markham et al.² and Sommer et al.³, this technique has been applied to the detection of single base changes and identification of specific alleles associated with disease in such diverse instances as cystic fibrosis^{4,5}, phenylketonuria³, apolipoprotein genotyping⁶ and HLA typing⁷. Larder et al. have applied this technique to examine AZT resistance acquired during chemotherapy, first, by characterizing the genetic mutations in the HIV RT gene that can be linked to *in vitro* resistance⁸, and more recently by applying this technique to the direct determination of the presence or absence of these mutations in patient blood samples⁹. Further work by his group has validated

and extended this approach ¹⁰⁻¹³.

In addition to the drugs currently available, a number of new agents are being developed and tested as HIV chemotherapeutic agents against both the HIV RT gene^{14,15} and other viral targets such as the integrase protein¹⁶, and the HIV protease¹⁷. It is expected that as the new agents and combination therapies are administered to patients, new mutations conferring resistance to these agents will also be discovered. It will be useful to monitor the appearance of resistant virus in patient populations in order to adjust the therapeutic regimes in use at the time and that is the intent of the ACTG's 244 protocol. Although the details of the assay described below are for the detection of the mutation at amino acid 215 that confers AZT resistance, this procedure is readily adaptable to the detection and monitoring of mutations at other locations within the viral RT gene simply by changing the primers used in the second (nested) PCR reaction and re-optimizing the PCR reaction conditions (if needed) to maximize sensitivity. Indeed, this has been accomplished at SRA for the 74 mutation associated with DDI resistance and for codon 184 for 3TC; our efforts in this regard are also documented elsewhere in this report. Our most recent incarnation of the 215 ARMS protocol for the detection of AZT resistant genotypes is described below.

ARMS Protocol

PCR Reactions - 215 Mutation Detection

We use A(35 mer) and NE1(35 mer) primers for the first set of cycles and the B and either 215M or 215W primers for the second set of cycles to detect mutant (resistant) or wild type (sensitive), respectively. These primers are identical to those described by Larder and Boucher (B. Larder, personal communication¹⁸). The primer sequences in use today are given below. The NE1(35), 215M and 215W are 5' biotinylated.

A(35)	TTGGTTGCACTTTAAATTTTCCCATTAGTCCTATT
NE1(35)	CCTACTAACTTCTGTATGTCATTGACAGTCCAGCT
B	GGATGGAAAGGATCACC
215M	ATGTTTTTTGTCTGGTGTGAA
215W	ATGTTTTTTGTCTGGTGTGGT

The PCR cycle part of the assay is identical whether the source material is plasma or tissue culture supernatants (viral RNA), or patient PBMCs or co-culture cells (proviral DNA). However, serum samples and samples treated with heparin have proven to be somewhat difficult to handle and 40 cycles has been the standard for the first cycle of amplification with those samples. Sample preparation steps are given for each substrate. New protocols for all steps of the ARMS assay are also

provided.

Sample Preparation: PBMCs or Co-cultured Cells

1. Thaw frozen cells at 37°C and transfer to a sterile 15 ml polypropylene centrifuge tube.
2. Wash once with 10 ml PBS (2000 rpm 15 min.). Decant supernatant after wash and discard.
3. Add lysis buffer (10 mM Tris 8.3, 50mM KCl, 2.5mM MgCl₂, 0.45% NP-40, 0.45% Tween 20, proteinase K at 120 µg/ml) and resuspend pellet well for a cell concentration of 7.5×10^6 cells/ml. Be sure to lyse a negative control with the cell samples. Vortex briefly.
4. Incubate at 55°C - 60°C for 1 hr. Vortex before transfer in next step.
5. Transfer to 1.5 ml screw-cap microcentrifuge tube.
6. Heat-inactivate the proteinase K by incubating the tubes at 95°C for 15 min.
7. Transfer tubes to ice. Store lysates either at 4°C (no more than overnight) or at -20°C for longer periods.

Sample Preparation: Viral RNA from Plasma

Preparation of Plasma from Whole Blood

1. Centrifuge the whole blood at approximately 200 X g (1400 rpm) in a tabletop centrifuge at 4°C.
2. Remove the supernatant, taking care not to disturb the cell layer.
3. Centrifuge the supernatant from Step 2 at approximately 1000 X g (4000 rpm) in a tabletop centrifuge at 4°C. The purpose of this step is clarification, to remove residual cells.
4. If the plasma is to be extracted immediately, maintain on ice. If it is to be stored, aliquot into 1.0 ml cryo-vials, and store at -70°C.

Pelleting of Virus

1. If the sample is frozen, thaw quickly at 37°C, then maintain on ice.
2. Add 0.5 ml PBS/BSA to 1.5 ml screw cap tube. Tubes should be pre-labelled

with specimen number.

3. Transfer 1 ml of plasma to each tube. Be sure to include negative control.
4. Pellet the virus by centrifugation at $12,000 \times g$ (approximately maximum speed in a typical microcentrifuge at 4°C for 1h.

RNA Extraction

1. Remove and discard supernatant from the 1.5 ml tube by decanting and removing as much of the supernatant as possible while the tube is inverted. Be careful not to disturb the pellet. Gentle tapping on clean gauze will help remove supernatant.
2. Add 800 μl Tri-Reagent (guanidinium/phenol). Vortex 15 s.
3. Allow to sit at least 5 min. at room temperature.
4. Add 160 μl CHCl_3 to each tube. Vortex 15 s.
5. Allow to sit at least 3 min. at room temperature.
6. Centrifuge at maximum speed (approximately $12,000 \times g$) in a microcentrifuge at 4°C for 15 min.
7. Remove the aqueous (upper, colorless) phase to a fresh tube.
8. Add 400 μl of cold isopropanol (IPA, 2-propanol) and 4 μl of 2.5 $\mu\text{g}/\mu\text{l}$ tRNA to each tube. Mix well by vortexing.
9. Maintain at -20°C overnight.
10. Centrifuge at maximum speed in a microfuge at 4°C for 15 min.
11. Decant the supernatant.
12. Wash the pellet with 1 ml of ice-cold 75% ethanol. Vortex briefly.
13. Centrifuge at maximum speed (approximately $12,000 \times g$) in a microfuge at 4°C for 15 min.
14. Decant the supernatant.
15. Air dry the pellet. Do not use a Speed-Vac.

16. Add Virus Lysis Buffer A (1% NP-40, 0.04 mg/ml tRNA, 0.4 U/ μ l RNasin, 2 mM DTT) for 55 μ l per 1 ml original specimen volume. Vortex.
17. Maintain at 42°C approximately 15 min. to fully dissolve the RNA, after which the extract should be stored at -70°C. The RNA should not be heated above 60°C until after reverse transcription, as RNasin in the Virus Lysis Buffer will be inactivated.

215 PCR Reaction Setup

A. DNA PCR:

The first set of PCR cycles uses A(35) & NE1(35) primers (NE1(35) primer is biotinylated) to produce a 805 bp fragment encompassing virtually all currently known drug resistance associated mutations in the HIV RT gene (amino acids 5-254 of RT).

PCR master mix:

7.1 μ l H₂O
22.4 μ l dNTP (280 μ M dNTP) (use dUTP only in the second PCR)
10.0 μ l 10X 215 PCR buffer
10.0 μ l Primers [A(35) & NE1(35); 250ng each.]

49.5 μ l store at -20°C if required.

Add Taq polymerase (Promega, 5 units/ μ l) when ready to use.

When ready to begin PCR, aliquot 50 μ l of PCR mix with the Taq polymerase added to each reaction tube.

Add 70.0 μ l of oil overlay (may be omitted for PE 9600 cycler)

Add 50 μ l of sample lysate

NOTE: In contrast to the PCR protocol in use for HIV detection in our laboratory, dUTP and UNG are not used in the first PCR of this nested set. The use of dUTP significantly reduces the discriminating power of the 215W and 215M primers used in the second PCR reaction.

The 10X 215 PCR buffer contains:

500 mM Tris 8.3
250 mM KCl
15 mM MgCl₂

1 µg/ml BSA

1. In the positive control lab, add positive controls (10⁴ sensitive and/or resistant cells) from freshly thawed stock dilutions to the appropriate PCR tubes.
2. Immediately carry the reactions to the cycler.

PCR Cycling Conditions for Perkin Elmer 9600 Cycler

First (Outer) PCR Reaction Cycling Conditions

1. 94°C 1' 15"
2. 94°C 30"
3. 55°C 30"
4. 72°C 2'
5. Repeat steps 2-4 for 18-30 cycles
6. Soak 72°C 10 min.
7. Soak 40°C

The precise number of cycles depends somewhat upon the expected number of infected cells. When testing co-cultured cells, 18 cycles is usually sufficient, due to the large number of infected cells in the population, whereas primary patient cells often require 30 cycles, while up to 40 cycles may be used to generate more product if needed for cloning and sequencing. Since there is no UNG in these reactions, they may be maintained at 25°C after last cycle until products are ready to be carried into second PCR. Yield of PCR products may be monitored by running a 10 µl aliquot of the first PCR reaction on an agarose gel. It should be noted that in cases of low numbers of virus, such as seen in patient PBMCs, no band may be visible after the first PCR. This is not necessarily an indication that the PCR failed however.

Reaction Setup for Second (Nested) PCR

The second (nested) PCR reaction utilizes the B and either 215W or 215M primers to discriminate between the wild type (AZT sensitive) or mutant (AZT resistant) genotype at amino acid 215 of the HIV-1 RT gene. The 215W primer recognizes either the Phe or Tyr mutant at amino acid 215 approximately equally.

1. Remove 10 µl of the **1st PCR reaction** and dilute 1:10 to 1:100 in water.

NOTE: The exact dilution can be varied to ensure clean discrimination between 215M and 215W primer products. In general, there is less than a 100X difference in the product yield between completely homologous primer/template combinations (sensitive virus DNA with 215W primer for

example) and mis-matched primer/template combinations (sensitive virus DNA with 215M primer) (Dr. Frank White, unpublished observations). Because of this, if the quantity of product transferred into the second PCR (B & 215M/215W) is too high, cross-reactive bands appear in both sensitive and resistant reaction lanes. In this case, it is necessary to either dilute the products of the first PCR reaction further and repeat the second PCR, or repeat the first PCR with reduced cycle numbers. This latter approach generally gives slightly cleaner results, but is also more time and labor intensive. The second PCR is set up exactly as the first, with the addition of 10 µl of first reaction product and 90 µl of master mix containing the B and 215M or 215W primers. Note that dUTP can be substituted for dTTP in this reaction without affecting the specificity or sensitivity of the PCR. Uracil-N-glycosylase (UNG) may then be added to facilitate contamination control, as is the standard procedure for HIV detection PCR reactions.

PCR Master mix for second PCR (B & 215M/215W primers)

10.0 µl	dNTPs (250 µM dNTP. May substitute dUTP for dTTP)
10.0 µl	10X 215 PCR buffer
10.0 µl	primers (B & 215W, 215M) at the ratio of 1:2 total of B+215M or W should equal 250 ng/reaction
3.0 µl	MgCl ₂ (25 mM stock, 2.25 mM final concentration)

33.0 µl Store at -20°C until needed.

Add 56.3µl of H₂O, 0.2 µl UNG (0.2 units, Epicentre Technologies) and 0.5 µl Taq polymerase (Promega, 5 units/µl) per reaction when ready to use.

When ready to begin PCR aliquot 90.0 µl total of the above mix to each reaction tube a 70.0 µl oil overlay (may be omitted for PE 9600 Cyclor) and 10.0 µl diluted products from first PCR.

NOTE: There are slight changes in the concentrations of some of the reaction components (2.25 mM MgCl₂) between the first and second PCR reactions. These conditions have been optimized to increase product yield of the second PCR reaction.

Second (Nested) PCR Reaction Cycling Conditions

1. 25°C 3'
2. 94°C 5'
3. 94°C 1'
4. 48°C 30"
5. 72°C 30"

6. Repeat steps 2-4 for 30-40 cycles. Soak at 72°C after last cycle until products are either stored (-20°C) or analyzed.

B. For RNA PCR:

1. 215 RT PCR Master Mix:

DEPC H ₂ O	3.2 µl
5X RT Buffer	10.2 µl
0.1 M DTT	2.0 µl
0.05 µg/µl NE1' primer	5.0 µl
10% NP-40	3.4 µl
25.0 mM dNTP's	1.0 µl

Total 24.6 µl/reaction

Store in -20°C freezer. Add 0.2 µl of RNasin (40 units/ml) and 0.2 µl of MMLV-RT per reaction when ready to use.

- Add 25 µl of each RNA sample to a 96 well plate.
- Overlay with 20 µl of mineral oil, seal and place in a heat block at 75°C for about 5 min. Immediately place on ice or at 4°C.
- Add 25 µl of RT mix to each well.
- Use either a 9600 cycler or manual transfer between heat blocks set for the following temperatures:

42°C 30 min.
99°C 5 min.
- Place on ice or at 4°C for at least one min.

* Be sure that the tubes are pressed firmly in the heat block to ensure even heating.

2. A' PCR master mix:

DEPC H ₂ O	35.5 µl
10X "215" Buffer	5.0 µl
A' primer (0.05 µg/µl)	5.0 µl
25 mM MgCl ₂	4.0 µl

Total 49.5 µl/rxn

Store Master mix at -20oC.

Add Taq Polymerase (2.5 units) in 0.5 µl/rxn to master mix before use.

When the RT reactions are completed, add 50 µl of A' PCR mix to each tube and cycle as indicated in the 1st DNA PCR. Follow with 2nd PCR also as described for DNA PCR.

PCR Product Analysis

Figure 1 illustrates the sequences generated by the first PCR reaction and the second, nested PCR reaction. The second PCR product is 210 base pairs in length and is highlighted in bold letters and is bordered by the B primer or forward primer and the 215 discrimination primers (the wildtype in this example). This sequence was generated from HXB2RT. The aa215 codon is indicated by the enlarged lettering. Following the second PCR, 20 µl of the products are analyzed on a 3% agarose gel run in 1X TBE containing EtBr. Results are seen as bands in the lanes corresponding to reactions containing either the B/215W (sensitive) or B/215M (resistant) primers. An example of a typical gel is shown in figure 2. A mixture of sensitive and resistant virus can be seen as bands in both lanes. However, this may also indicate that the second PCR reaction was overloaded with product from the first PCR. To eliminate this problem, the number of cycles for the first PCR can be reduced (from 30 to 15-22) and/or the dilution of products from the first PCR increased from 1:10 or 1:100 to 1:1000 or even 1:100,000 if needed to produce a single band from one or the other PCR reaction.

Application of the 215 PCR Assay to Clinical Samples

To date, over **900 patient PBMC samples** and more than **1500 patient plasma (RNA), serum or CSF samples** have been analyzed with 215 PCR assay as improved at SRA Technologies. SRA has been validated by the ACTG as a center for 215 mutational analysis by its participation in the QC validation program in support of WRAIR' ongoing collaboration with the ACTG. The ARMS assay is now being applied to the ACTG Clinical protocol number 244 to determine its usefulness in the management of AIDS patients on antiviral therapy. In addition to protocol 244, the ARMS assay for 215 has been applied to recent studies of plasma or serum viral RNA in seroconvertors (data not shown). These studies, performed for WRAIR in

Figure 1

Products of the First and Second PCRs of
the ARMS Assay for aa215 of RT

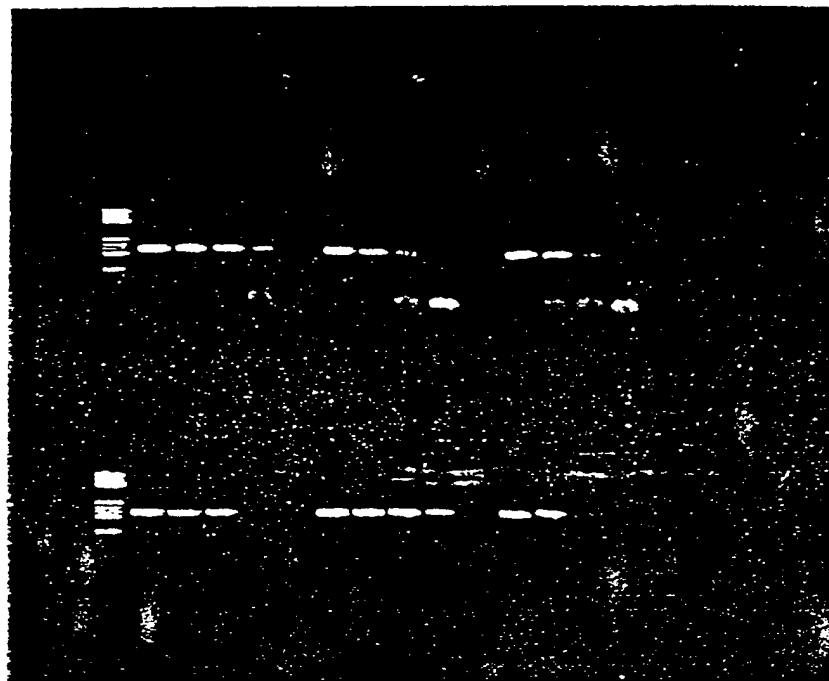
```

1 CCC ATT AGC CCT ATT GAG ACT GTA CCA GTA AAA TTA AAG CCA GGA ATG
  GGG TAA TCG GGA TAA CTC TGA CAT GGT CAT TTT AAT TTC GGT CCT TAC
49 GAT GGC CCA AAA GTT AAA CAA TGG CCA TTG ACA GAA GAA AAA ATA AAA
  CTA CCG GGT TTT CAA TTT GTT ACC GGT AAC TGT CTT CTT TTT TAT TTT
97 GCA TTA GTA GAA ATT TGT ACA GAG ATG GAA AAG GAA GGG AAA ATT TCA
  CGT AAT CAT CTT TAA ACA TGT CTC TAC CTT TTC CTT CCC TTT TAA AGT
145 AAA ATT GGG CCT GAA AAT CCA TAC AAT ACT CCA GTA TTT GCC ATA AAG
  TTT TAA CCC GGA CTT TTA GGT ATG TTA TGA GGT CAT AAA CGG TAT TTC
193 AAA AAA GAC AGT ACT AAA TGG AGA AAA TTA GTA GAT TTC AGA GAA CTT
  TTT TTT CTG TCA TGA TTT ACC TCT TTT AAT CAT CTA AAG TCT CTT GAA
241 AAT AAG AGA ACT CAA GAC TTC TGG GAA GTT CAA TTA GGA ATA CCA CAT
  TTA TTC TCT TGA GTT CTG AAG ACC CTT CAA GTT AAT CCT TAT GGT GTA
289 CCC GCA GGG TTA AAA AAG AAA AAA TCA GTA ACA GTA CTG GAT GTG GGT
  GGG CGT CCC AAT TTT TTC TTT TTT AGT CAT TGT CAT GAC CTA CAC CCA
337 GAT GCA TAT TTT TCA GTT CCC TTA GAT GAA GAC TTC AGG AAG TAT ACT
  CTA CGT ATA AAA AGT CAA GGG AAT CTA CTT CTG AAG TCC TTC ATA TGA
385 GCA TTT ACC ATA CCT AGT ATA AAC AAT GAG ACA CCA GGG ATT AGA TAT
  CGT AAA TGG TAT GGA TCA TAT TTG TTA CTC TGT GGT CCC TAA TCT ATA
433 CAG TAC AAT GTG CTT CCA CAG GGA TGG AAA GGA TCA CCA GCA ATA TTC
  GTC ATG TTA CAC GAA GGT GTC CCT ACC TTT CCT AGT GGT CGT TAT AAG
481 CAA AGT AGC ATG ACA AAA ATC TTA GAG CCT TTT AGA AAA CAA AAT CCA
  GTT TCA TCG TAC TGT TTT TAG AAT CTC GGA AAA TCT TTT GTT TTA GGT
529 GAC ATA GTT ATC TAT CAA TAC ATG GAT GAT TTG TAT GTA GGA TCT GAC
  CTG TAT CAA TAG ATA GTT ATG TAC CTA CTA AAC ATA CAT CCT AGA CTG
577 TTA GAA ATA GGG CAG CAT AGA ACA AAA ATA GAG GAG CTG AGA CAA CAT
  AAT CTT TAT CCC GTC GTA TCT TGT TTT TAT CTC CTC GAC TCT GTT GTA
625 CTG TTG AGG TGG GGA CTT ACC ACA CCA GAC AAA AAA CAT CAG AAA
  GAC AAC TCC ACC CCT GAA TGG TGT GGT CTG TTT TTT GTA GTC TTT
670 GAA CCT CCA TTC CTT TGG ATG GGT TAT GAA CTC CAT CCT GAT AAA TGG
  CTT GGA GGT AAG GAA ACC TAC CCA ATA CTT GAG GTA GGA CTA TTT ACC
718 ACA GTA CAG CCT ATA GTG CTG CCA GAA AAA GAC
  TGT CAT GTC GGA TAT CAC GAC GGT CTT TTT CTG

```

Figure 2

Typical Gel from ARMS Assay for Genotypic Resistance or Sensitivity to AZT



<u>Lane #</u>	<u>Description</u>	<u>Result</u>
1	Molecular Markers	
2	Specimen A 1:1000 Dilution	
3	Specimen A 1:10000 Dilution	
4	Specimen A 1:100000 Dilution	Sensitive
5	Specimen A 1:1000000 Dilution	
6	Space	
7	Specimen B 1:1000 Dilution	
8	Specimen B 1:10000 Dilution	
9	Specimen B 1:100000 Dilution	Resistant
10	Specimen B 1:1000000 Dilution	
11	Space	
12	Specimen C 1:1000 Dilution	
13	Specimen C 1:10000 Dilution	
14	Specimen C 1:100000 Dilution	Mixture
15	Specimen C 1:1000000 Dilution	

collaboration with laboratories in Switzerland, provided some of the earliest evidence for the transmission of AZT resistant HIV-1 (see publication and abstract citations in the Appendix to this report). SRA has also performed more than 180 assays for DDI mutations at codon 74 in the RT gene.

Additional ARMS Assay Development

Development of quantifiable RNA controls for the 215 ARMS assay.

One problem with the ARMS assay for the detection of the 215 mutation is the lack of standards to be run along with the test samples. The initial assay relied on viral stocks generated by infecting both wild type and mutant virus *in-vitro*. The problem with using this control is the potential variability in viral concentration as well as sequence. This prevented direct comparison of the assays between standards. In an attempt to address this problem we developed controls based on RNA, instead of the more variable patient cells. In this scenario actual synthesized RNA either incorporating the mutation or not would be used in the assay. The synthetic RNA could be quantitated spectrophotometrically. The advantage will be the ability to calculate an exact copy number of RNA template which will be used in the 215 assay. In addition, using this controlled template we will be able to better monitor the overall sensitivity of the assay and be alerted quicker if any degradation in the quality of the assay occurs.

Development of the RNA 215 control involved multiple steps. The first was generation of two recombinant plasmids containing either the 215 mutation or wild type. Viral HIV-1 stocks of both the resistant and sensitive phenotype were used to generate cDNA as described above. The cDNA was used as a template for PCR using the 215 primers A' and NE1'. The two resulting 750 bp fragments were subcloned into a plasmid vector containing a RNA transcription start site. The plasmids were grown and confirmed for the presence of the appropriate codon by sequencing. Once shown to be correct the plasmids were used as templates. RNA was synthesized by *in-vitro* transcription from the plasmid vector containing the region of HIV RT defined by primers "A" and "NE1". RNA was made from both a wild type and 215 mutant plasmid. The construct of the plasmid allowed the use of both the SP6 or T7 transcription promoters (located on either side of the RT insert) from each clone. Spectrophotometric analysis revealed that we had a large yield of RNA from both plasmids. The T7 promoter gave a larger yield (186ug) while SP6 yielded (121ug). Based on a RNA size of 800bp this calculates to 4.3×10^{12} and 3.0×10^{12} RNA copies. Qualitatively the RNA appeared of good quality by gel electrophoresis and in range with the spectrophotometer measurements. RNA was aliquoted and prepared for further testing.

Results of Testing synthetic RNA controls

Synthetic control RNA both containing and lacking the 215 mutation was tested in the ARMS 215 assay. The assay was performed as previously described. RNA was diluted up to 1:1,000,000 with the best results obtained with the 1:100,000 dilution. Template too concentrated lost all ability to discriminate when subject to the second PCR using the 215 specific primers. It was also observed that the *in vitro* transcription product was not completely specific, as the negative control transcripts from either T7 or SP6 promotor gave a certain amount of product but always less than the properly oriented RNA transcript. A range of concentrations was established which could be used as both positive and negative controls for the wild type and mutant PCR. These synthetic RNA controls have now been incorporated into the 215 ARMS assay as controls.

Development of ARMS assay for detection of the 184 HIV RT mutation.

Drug combination therapy for treatment of HIV infection while having beneficial effects also has led to the emergence of new resistant strains of HIV. This phenomenon has been explored by studies of the combination of AZT and 3TC (Larder et al. , 1995 Science). This study expanded on earlier work that showed that resistance to nucleoside analog 3TC is associated with a mutation in the RT at position 184 (Met to Val) (Schuurman et al, 1995, J Infect Dis). In order to assay for the presence of this mutation in HIV infected patients we developed an ARMS assay similar to the 215 assay for RT position 184. The basis of this assay is identical to the 215 ARMS assay except for the primers used for amplification. We synthesized 3 primers corresponding to a forward 184 mutant primer , a forward wild type ,and a common wild type reverse primer.

Forward 184M(Mutant)

5' CAG-ACA-TAG-TTA-TCT-ATC-AAT-ACG 3'

Forward 184W(wild type)

5' CAG-ACA-TAG-TTA-TCT-ATC-AAT-ACA 3'

Reverse 184Rev

5' GTA-ATC-CCC-ACC-TCA-ACA-GA 3'

These primers are used in place of the second set of primers of the 215 assay, after the initial amplification from primers A/ NE1. Controls for the 184 mutation were obtained from Dr. John Mellors. The controls consisted of patient plasma containing both 184 mutant and wild type virus. Our initial PCR showed that the primers were able to PCR a fragment of the appropriate size (123bp). This first PCR (annealing temp of 48°C) was unable to distinguish between the mutant and wild type. Using

the four samples with defined genotypes we next raised the annealing temperature to 50°C, which again gave bands, but with no discrimination. The next PCR was performed was conducted at a temperature of 52°C and the template (A/NE1 first PCR) was diluted serially to a dilution of 1:10,000. In this reaction we were able to see discrimination of the wild type primers from the mutant 184 template. Using PCR annealing conditions for the second PCR of 52°C the 184 ARMS assay was able to routinely distinguish the 3TC resistant from the sensitive virus.

Efforts to Develop a Quantitative Assay for Mutations at The 215 Codon

One major limitation of the currently used agarose gel based assay is that it does not allow accurate evaluation of patient samples containing mixtures of resistant and sensitive virus. Theoretically, the presence of a mixture of resistant and sensitive virus in a patient sample would produce PCR products from both the resistant and sensitive primer sets. These would be seen as bands appearing in both sets of lanes on a gel. Due to incomplete inhibition of primer extension from mismatched primers (such as the 215W primer hybridized to a resistant virus) however, it is possible to produce diagnostic bands of the gel from both the sensitive and resistant PCR reactions from samples that contain only one species of virus by simply overloading the second PCR reaction with product from the first (A & NE1 primer set) PCR reaction. Initial efforts to improve on this rather subjective procedure for analysis were not very successful. They included attempts to quantitate products of the second PCR using biotinylated and fluorescently tagged primers. Readout was on Molecular Dynamics FluorImager™ and both gel and capture plate formats were studied. Though product differentiation was possible, quantitation proved problematic and efforts in this direction were curtailed.

We pursued additional modifications to the 215 protocol with the intention of increasing the sensitivity of the assay when analyzing plasma RNA samples, and to improve the quantitative ability of the assay. It was hoped that a new assay procedure, available through Perkin-Elmer, the TaqMan™ PCR procedure might prove more fruitful and studies, in collaboration with Perkin-Elmer's applications group were started, but the approach proved too expensive at that time. SRA (using its own funds independent of the contract) also began a series of investigations to develop quantitative mutation assays with general applicability. Both nucleotide addition and fluorescently tagged primer assays were evaluated using the 377 automated sequencer and GeneScan software from Perkin-Elmer. These independent studies led to the development of a quantitative microsequencing procedure (QMASSM) that can be made available to the government on request.

Diagnostic DNA Sequencing

Despite the effectiveness of the 215 PCR protocol described in the previous section, it is limited in that it can only be used where the site of mutation is known, and then only when the surrounding sequence is conserved sufficiently to ensure efficient primer hybridization. For new drugs, where the site of the resistance-conferring mutation is not well characterized, or for mutations occurring in hypervariable regions, diagnostic sequencing is the only method that can provide useful genotypic information. During the first two years of the contract a manual sequencing procedure was developed, underwent a number of modifications and was subsequently supplanted by an automated procedure that employs an ABI 377 sequencer based on the use of fluorescence.

With the addition of new drug therapies focusing on both the reverse transcriptase and protease genes of HIV, a multitude of new resistance mutations have appeared. These mutations confer resistance to the Nucleoside analogs (AZT, ddC, ddI), non-nucleoside reverse transcriptase inhibitors (NVP, DVP), and the newly developed protease inhibitors (SQV, VX, RTV, NEL). The abundance of mutations and the combinations of therapies make sequencing the preferred choice for antiviral genotyping. SRA, internally and together with outside collaborators (CPCRA), have established the following protocol for sequencing both the protease and RT (to codon 250) of HIV from patient plasma.

Sequencing Step-by-Step Procedure

SEQUENCING Protocol for THE PROTEASE AND REVERSE TRANSCRIPTASE GENES OF VIRION-ASSOCIATED HIV-1 RNA

A. RNA Purification(Modified QIAamp HCV RNA kit procedure)

1. Transfer 500 ul of plasma into a 1.5 ml microcentrifuge tube and centrifuge for 14,000 x g for 30 min. Or 125,000 x g for 10 min to pellet the virus.
2. Discard the supernatant, and resuspend the virus pellet in 140 ul of water.
3. Add 560 ul prepared Buffer AVL to the sample. Mix by vortexing.
4. Incubate at room temperature for 10 min.
5. Add 560 ul of ethanol (96-100%) to the sample, and mix thoroughly by vortexing. Place a QIAamp spin column in a 2-ml collection tube.
6. Apply 630 ul of the sample solution to the spin column and centrifuge at

6,000 x g for 1 min. Transfer the spin column to a clean collection tube.

7. Apply the remainder of the sample solution to the spin column, and centrifuge at 6,000 x g for 1 min. Transfer the spin column to a clean collection tube.
8. Add 500 ul of buffer AW to the spin column, and centrifuge at 6,000 x g for 1 min. Transfer the spin column to clean collection tube.
9. Add another 500 ul of buffer AW to the spin column, and centrifuge at full speed (approx. 20,000 x g) for 3 min.
10. Transfer the spin column to a 1.5 ml microcentrifuge tube, and add 50 ul of 80 C-heated Rnase-free water to the spin column. Centrifuge at 6,000 x g for 1 min to collect the RNA.

B. Reverse Transcription-First Round PCR

1. For each sample, prepare the following reaction mix (or its equivalent) using Life Technologies Superscript One-Step Reagent Kit:

25.0 ul 2x Reaction Buffer
5.0 ul 5 mM magnesium sulfate
0.5 ul primer RT21 (50 pmol/ul)
0.5 ul primer MAW-26(50 pmol/ul)
1.0 ul Superscript-TaQ Enzyme Mix

2. Dispense 32 ul of reaction mix into PCR reaction tubes.
3. Add the 18 ul of test sample into a reaction tube.
4. Place the reaction tubes on the thermal cycler and run using the following parameters:

1 cycle of: 45 C for 30 min
 94 C for 2 min
40 cycles of: 94 C for 15 sec
 55 C for 20 sec
 72 C for 2 min
1 cycle of: 72 C for 10 min
5. At the completion of the cycling, the samples can be stored at 4 C for 24 hr or at -20 C or lower for longer periods.

C. Second Round PCR

1. For each sample, prepare the following reaction mix (or its equivalent):

10.0 ul PcR Buffer II
10.0 ul 25 mM magnesium chloride
0.6 ul 25 mM dNTPs
0.2 ul primer PRO-1 (50 pmol/ul)
0.2 ul primer RT20 (50 pmol/ul)
0.5 ul Taq DNA polymerase
73.5 ul water

2. Mix the reaction mix thoroughly and pipette 95 ul of mix to separate PCR reaction tubes .
3. Pipette 5 ul of the first round PCR product into a reaction mix tube.
4. Place the reaction tubes on the thermal cycler and run using the following parameters:

35 cycles of: 94 C for 15 sec
63 C for 20 sec
72 C for 2 min
1 cycle of: 72 C for 10 min

5. At the completion of the cycling, the samples may be analyzed immediately or stored at 4 C or lower.

D. Gel Electrophoresis

1. Ten ul of the PcR reactions will be analyzed on an appropriate agarose gel, using standard laboratory procedures, to verify that amplifications were successful.

E. PCR Product Preparation

1. PCR products obtained from successful amplifications will be purified using common protocols for PcR product purification (e.g. Qiagen, Centricon). Alternatively, products can be diluted in water at a dilution factor of 1:3 to 1:5, depending on the intensity of the band, and added directly to the sequencing reaction (Step G).

F. DNA Quantitation After Purification

1. If the PCR products are purified using a common protocol, 1/5 of the resulting DNA (e.g. 10 ul from a 50 ul Qiagen purification) will be diluted in water to a total volume of 50 ul.
2. The DNA dilution is measured at 260 nm on an appropriate spectrophotometer.
3. The concentration of DNA is calculated manually or by using spectrophotometer software.
4. The original DNA samples are adjusted to approximately 25 ng/ul.

G. Sequencing Reactions

For this study, the following primers will be used (alternative selections will be used when a first choice primer fails in a given sample):

Protease Gene: DSPR, USPR-3 (PSR2, proseq)

RT Gene: RT-a, HXB2-89, and Brev (HXB2-88, B, RT-y, RT-z)

1. Sequencing reactions mixes are prepared ON Ice in the following manner using custom-made dye-labelled sequencing primers (at 0.4 pmol/ul) and ABI Dye Primer Core Kits:

Microliters of reagent per sample per dye primer:

	<u>A mix</u>	<u>C mix</u>	<u>G mix</u>	<u>T mix</u>
5x reaction buffer	1	1	2	2
dye-labelled primer	1	1	2	2
dd/dNTP mix	1	1	2	2
*diluted Amplitaq FS	1	1	2	2

*diluted Amplitaq FS (per sample per primer):

1 ul amplitaq FS

1 ul 5X reaction buffer

5 ul water

2. Pipette the prepared reaction mix and the prepared PCR product into PCR reaction tubes ON ICE:

	<u>A</u>	<u>C</u>	<u>G</u>	<u>T</u>
Sample A ul reaction mix	4	4	8	8
ul PCR product	1	1	2	2
Sample B ul reaction mix	4	4	8	8
ul PCR product	1	1	2	2
Sample C ul reaction mix	4	4	8	8
ul PCR product	1	1	2	2

- The reactions are then run on a thermal cycler using cycling parameters optimal for each primer.

For dye primers B, Brev, RT-b, HXB2-88, HXB2-89, RT-y, RT-z, RT-a, DSPR and USPR-3:

1 cycle of: 98 C for 5 sec
 30 cycles of: 96 C for 5 sec
 68 C for 1 min

For dye primers PSR-2 and proseq:

1 cycle of: 98 C for 5 sec
 30 cycles of: 96 C for 10 sec
 60 C for 5 sec
 70 C for 1 min

- Following the completion for the cycling run, the reactions are either processed immediately or stored at -20 C.

H. Processing of Sequencing Reactions

- For each sample, the contents of each of the 4 reaction tubes (A, C, G, T) are pooled together.
- The pooled reactions are then added to a 0.5 ml microcentrifuge tube that contains 100 ul of 95% ethanol to precipitate the DNA.
- The tubes are held on ice for 15-20 min, then centrifuged at 12,000-14,000 x g for 20-30 min in a microcentrifuge to pellet the DNA.
- The supernatant is discarded, and the pellet (not visible) is dried using a Speed-Vac (or equivalent).

5. The dried reactins are ready to be run on a sequencing gel, or can be stored (dry) at -20 C.

A. Template Preparation

It is recommended that PCR products be generated by a method called "Ampliwax Hot Start". Better sequencing results have been obtained using "Hot Start" PCR products because fewer non-specific products are generated.

1. Purify template by placing into the retentate cup of a Microcon 100 with 300 μ l TE buffer.
2. Microcentrifuge at 3,000 RPM's for 9 minutes.
3. Empty the waste in the centrifuge tube and repeat step #2 two more times, each time washing with 400 μ l of TE buffer.
4. After the last wash, remove the cap and place retentate cup into a clean catch tube and turn upside down, so that the sample reservoir is inside the centrifuge tube. Centrifuge at 1,000 RPM's for 5 min.

B. Sequencing Reaction

1. Label four 0.5 ml centrifuge tubes A,G,C and T. Place 3 μ l of ddATP termination mix into the tube labeled A, and do the same for G,C and T tubes. Store on ice.
2. In a separate 0.5 ml centrifuge tube, combine the following:

1/20 volume of purified PCR product
2 pmole Primer
4 μ l 10x sequencing buffer
1 μ l a ^{33}P dATP (10 μCi)
1 μ l Exo- PFU polymerase (2.5 U)
Bring volume to 26 μ l with ultra pure water
4 μ l DMSO

final volume = 30 μ l

Mix well by pipetting and a brief spin in the centrifuge. Keep on ice.

3. Immediately aliquot 7 μ l of the reaction mixture from step #2 into each of the 4 termination tubes containing 3 μ l of their appropriate ddNTP. Mix thoroughly, making sure reaction mix and dideoxynucleotide mix

are at the bottom of the tube.

4. Overlay the reaction with 15 μ l mineral oil. Briefly centrifuge.

5. Cycle reactions as follows:

Denature at 95°C for 5 minutes
95°C for 30 s
60°C for 30 s 30 cycles
72°C for 1 min.
Hold at 72°C

***Note:** Reactions should not be held for more than 5 minutes at 72°C, degradation can occur. The cycling takes approximately 1 1/2 - 2 hours.

6. At the end of the cycling procedure, add 5 μ l of stop solution to each reaction tube and immediately place on ice.

7. When ready to load gel, denature samples at 75-80°C for 5 minutes, place on ice and load 3 μ l on gel. Otherwise, samples can be stored at 70°C until ready for use.

Sequence Data Analysis

Manual DNA sequence information is acquired with a Howtek Scanmaster 3+ flat bed scanner used to digitalize the entire 35X43 cm film of the sequencing gel. Using input data from digitized films the Millipore/BioImage analysis software SRA currently runs on a Sun Sparc 10 workstation can automatically define the sequencing reaction lanes and perform automatic base calling using neural network algorithms for increased accuracy. These advanced algorithms can be "trained" to improve base-calling accuracy the more they are used. In addition, the software allows quantitation of band intensity from the sequencing gel, facilitating determination of mixed population at a particular base location based in the sequencing gel information.

Table 1

Amino Acid

Sample	18	40	62	98	100	108	136	150	160	180	181	192	205	214	229
HIVHXB2	GGC (Gly)	GAG (Glu)	GGC (Ala)	GCA (Ala)	TTA (Leu)	GTA (Val)	AAC (Asn)	CCA (Pro)	TTC (Phe)	TAT (Tyr)	GAC (Asp)	CTG (Leu)	CTT (Leu)	TGG (Trp)	
DRUG M RESIST.			GGC (Ala) GTC (Val)	GGA (Gly) GCA (Ala)	ATA (Ile)	AAT (Asn)	-						CTT (Leu) TTT (Phe) CTA (Leu)		
DRUG N RESIST.		GAA (Glu)			ATA (Ile)										
DRUG O RESIST.					ATA (Ile)				TTT (Phe)				TTT (Phe) CTT (Leu)	TGG (Trp) TTG (Leu)	
DRUG P RESIST.	GGC (Gly) GGT (Gly)									TGT (Cys)					
DRUG Q RESIST.		GAA (Glu) GAG (Glu)						-		TGT (Cys)	GAC (Asp) GAT (Asp)				
IIIB							AAC (Asn) AAT (Asn)	-							

This system is currently connected to our LAN (described in the reports section) and can access other computers both in-house and through the Internet. The system can also interact with other molecular biology software packages, such as the DNASTar Lasergene system currently running on a Mac Ilci with Genbank on CD-Rom. Custom filters have been provided to directly access the Mac format DNASTar files for use on the SUN system. These capabilities will provide easy access to the sequence data generated in our laboratories, and the information generated can easily be included in requested reports.

During the three base contract years SRA's manual sequencing protocols underwent a number of procedural modifications intended to reduce error rates frequently associated with the use of *Taq* DNA polymerase. When using PCR based sequencing techniques, several potential problems may arise. Several studies have examined the error rate of *Taq* DNA polymerase when used in a PCR assay¹⁹⁻²¹. SRA now employs the recently introduced thermostable DNA polymerase (*Pfu* DNA polymerase, Stratagene Inc.), which is possessed of a 3'-exonuclease proofreading activity, that significantly reduces the chance of PCR induced errors in sequence determination²². *Pfu* polymerase has now been substituted for NEB CircumVent™ in all DNA manual sequencing reactions at SRA.

During the past year, SRA performed sequencing in support of WRAIR's collaborations with the ACTG. AT the COR's request we have become active members of the ACTG's virology committee's sequencing "Swat team, " and have sequenced a number of QC panels in support of this effort. Halfway through the fiscal year, SRA Technologies acquired a Perkin-Elmer ABI Prism 377 automated DNA sequencer, making us one of the first sites to obtain the new model. The most recent of the ACTG QC samples were sequenced by both manual and automated methods, yielding identical results. SRA's automated sequencing capabilities permit rapid throughput of DNA sequencing samples and larger batch sizes. During the three base years of the contract SRA staff manually **sequenced the RT gene from more than 41 Isolates or cell pellets representing more than 30 Kilobases** and have been **validated as an ACTG sequencing site** by virtue of our participation in QC validations on WRAIR's behalf. Table 1 presents an example of a manual sequencing run. These data were originally presented in the FY93 annual progress report. All future efforts will utilize the more efficient automated approach.

While we initially performed manual sequencing we have successfully switched exclusively to automated sequencing using the ABI 377XL automated DNA sequencer. The advantages of this system is higher throughput and more reliable runs. Several hundred sequences representing thousands of sequenced bases have been produced using our automated approach. An additional benefit is the "electronic " nature of the data. ABI provides propriety software for the analysis and alignment of the sequence data. This software was used for some of the initial reports allowing for crude sequence comparisons. Part of the problems with the ABI

software is that it is a generic package for all sequencing projects. In order to generate a report more tailored to HIV genotyping and more interpretable to clinicians we joined with the Data Management Department of SRA to develop a semi-automated reporting system for sequence data. The resulting software and data output are reviewed in section 4 below (Data Management Section) and an example of the output can be found in Appendix I.

Viral Burden Assays

SRA began development of a quantitative RT-PCR at the start of the first contract year. This assay, an avidin-biotin capture plate-based procedure, required extensive evaluation and validation and proved, until recently, too variable and inconsistent to be employed for any serious evaluations of viral burden. The assay has since been modified and is available to serve as a backup to the widely utilized Roche Amplicor kit and the more laborious, but exceedingly sensitive procedure developed by Dr. Vahey of the Henry Jackson Foundation. At the beginning of the last base contract year, it was decided that WRAIR would provide SRA Technologies with an older model a phosphorimager (donated by Dr. Vahey). The intention was to encourage transfer of this technology to SRA where a production level procedure might be put in place to satisfy WRAIR's requirements for large-scale viral burden analyses. Previously, we had been supporting Dr. Vahey's work by preparing viral RNA from patient plasma, which was then sent to the Jackson Foundation for further analysis. During November and December of 1994 (FY95), two members of the SRA staff were trained in the assay, and the phosphorimager was transferred to SRA in late December. It took SRA a while to get the assay up and running, for several reasons. The phosphorimager was damaged while it was in shipment to SRA, and it took several weeks for it to be repaired. Second, the RNA transcript that was used for the

Amplicor HIV Monitor Calculations

Control ID: 14887

Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.950160	.441	x 125	55.125	15.841	x66	x40	41819
	.696	x 5	3.48				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.950113	-.029	x 25	ND		x	x40	ND
	.789	x 5	3.945				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB001	.482	x 625	301.25	91.985	x66	x40	242840
	.655	x 5	3.275				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB002	.916	x 25	22.9	10.701	x66	x40	28250
	.428	x 5	2.14				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB003	.484	x 25	12.1	3.380	x66	x40	8923
	.716	x 5	3.58				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB014	-.029	x 625	ND		x	x40	ND
	-.023	x 5	ND				
	QS OD	QS DF	OD*DF				

Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB017	.451	x 25	11.275	5.113	x66	x40	13499
	.441	x 5	2.205				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB001	.491	x 625	306.875	77.887	x66	x40	205622
	.788	x 5	3.94				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB002	.267	x 125	33.375	9.646	x66	x40	25465
	.692	x 5	3.46				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB003	.433	x 25	10.825	2.887	x66	x40	7621
	.75	x 5	3.75				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB014	..028	x 625	ND		x	x40	ND
	-.021	x 5	ND				
	QS OD	QS DF	OD*DF				
Sample	HIV O.D.	HIV DF	(OD*DF)	Total HIV/ Total QS	QS	Sample DF	HIV Copies/ml
760.VB017	.679	x 25	16.975	5.330	x66	x40	14070
	.637	x 5	3.185				
	QS OD	QS DF	OD*DF				

For Research Use Only.

Table 2

standard curve did not work, and had to be prepared a second time. Finally, a contamination problem was discovered that previously had been difficult to detect. This contamination problem was probably caused, in part, by the large volume of PCR experiments done at SRA. It appears that this contamination stemmed from our use of UNG in most PCR reactions. Since UNG is not used in the Viral Burden Assay, it is more sensitive to carryover contamination.

We assayed over 50 samples for viral burden utilizing the Liquid Hybridization procedure. In addition, **we have extracted well over 1100 samples for viral burden analysis** for Dr. Vahey's laboratory during the period covered by this report.

It was finally decided that viral burden studies would be accomplished using the Roche Amplicor HIV-1 quantitative PCR assay. Support for the liquid hybridization protocol was terminated and the equipment loaned to us returned. We currently have four laboratory scientists trained and certified by Roche in use of the Amplicor viral burden assay. We have analyzed **the viral burdens of 250 heparinized CPCRA samples** using the Amplicor procedure supplemented by a rather laborious extraction procedure employing silica. The Roche Amplicor system was turned over to SRA's DoD clinical diagnostic contract for all future work.

In support of this effort, **SRA has developed a computer program**, utilizing Oracle and its laboratory information management system **that automates parts of the viral burden analysis**. A sample of this software's output is shown in Table 2 above.

2. Cellular Phenotype Working Group

The Cellular Phenotype Working Group investigated three major areas during the three base contract years. These included; 1) developing and validating efficient phenotypic assays to support evaluation of neutralizing antibodies in vaccinees, 2) providing newly expanded and titrated stocks of primary HIV-1 isolates for WRAIR investigators along with phenotypic characterizations of isolate cytopathicity in support of various WRAIR protocols and 3) developing and implementing *in vitro* systems to evaluate antiviral genes for the treatment of HIV disease. The progress made during the contract period in each of these areas is reviewed below.

Development and Optimization of Virus Titration and Neutralization Assays

The majority of virus neutralization studies reviewed here were completed during the contract's first two years. No neutralization work was performed in the last of the contract's three base years. Virus neutralization assays may be employed to identify and differentiate virus, as well as to determine the host immune responsiveness to a specific viral infection and/or vaccination with various viral protein(s). Although identification of serum antibodies which inhibit viral infection *in vitro* may be a

useful marker of protective immunity for some viruses *in vivo*²³, the significance of neutralizing antibodies in influencing clinical outcome in HIV infected individuals is not well understood.²⁴⁻³⁰ Currently, there are a number of assays being used to evaluate the effect of antibody on HIV replication. Originally, most studies utilized immortalized cells (e.g., H-9, MT-2, etc) that exhibited susceptibility to one or more laboratory strains of HIV (IIIb, RF, MN, etc). Susceptibility was usually evaluated by the production of viral markers and/or the induction of cytopathic effects (CPE).³¹⁻³⁵ However, most field isolates of HIV (i.e., low passaged, patient isolates) infect immortalized cells with very low efficiency, thus these assays are of limited value in assessing the neutralizing antibody titers of a patients sera to clinical isolates.

During the first contract year considerable effort was placed in defining the conditions for optimum growth of HIV in PBMCs and a number of particularly sensitive leukopacks were chosen for subsequent use in all studies. We developed procedures for the evaluation of leukopack-derived donor PBMCs for sensitivity to HIV infection. This was important for two reasons. First, the assays we were required to develop were short term systems usually of four days duration. This necessitated the production of measureable levels of HIV p24 (the endpoint for the majority of studies) in a short span of time. Secondly, many of the clinical isolates employed in this work were slow growers, producing only low levels of p24 over the required four day period of culture. Thus use of only the most virus sensitive PBMCs was required. Using a pre-neutralization virus titration procedure, developed during the past year, we analyzed a number of donor leukopack-derived PBMCs for sensitivity to virus infection by slow/low (from early stage patients) and rapid/high variants of HIV (from late stage AIDS patients) on donor cells. The best donors, i.e. those that provide the highest titers in the shortest period of time are selected, in some cases pooled, and whenever possible, used throughout the entire process of virus titration, characterization and neutralization (or other immunoassay analyses) as required.

The data in Table 3 illustrate SRA's use of the aforementioned pre-neutralization titration protocol for evaluation of cryopreserved PBMCs obtained from eight normal donors. The cells were tested in a single experiment against four separate isolates of HIV-1. These isolates were donated by investigators at the Walter Reed Army Institute for Research (WRAIR) and the Henry M. Jackson Foundation (HMJF) who classified them as rapid/high (9881 and 873b) or slow/low (8871 and 4026).

Table 3
EFFECT OF DONOR PBMCs ON VIRUS TITER

PBMC DONOR	VIRUS ISOLATE			
	4026	8736	8871	9881
13108	1024*	2048	12944	131072
14486	NT	512	648	10369
14557	2048	1024	12944	>131072
14659	8192	3236	65536	>131072
16952	20738	1024	4096	>131072
17679	128	2048	676	32768
18072	809	648	12944	103552
18251	1024	512	5404	51776

* TCID₅₀ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Kärber.

The data suggest that a remarkable variability exists between donor PBMCs as regards their ability to support replication of these isolates. The reasons for this variability are not immediately evident.

As noted above, it is SRA's intention to utilize single leukopack donor-derived PBMCs for all phases of neutralization study including virus titration and neutralization. To ensure that sufficient numbers of PBMCs are available for *in vitro* studies we have evaluated a single donor's multiple bleeds, individually and as cell pools, for sensitivity to infection with rapid/hi (9881) and slow/low (4971) variants of HIV. The data generated in this study are summarized in Table 4 below. In one experiment leukopacks 20841, 21480 and 18251 were tested individually for sensitivity to virus in a pre-neutralization titration. Leukopacks 20841 and 21480 were clearly more sensitive to virus replication at days 4 and 7 when compared to 18251.

Table 4

EFFECT OF LEUKOPACK POOLS ON VIRUS TITERS

<u>Leukopack Pools</u>					
<u>Exp 1</u>			<u>Exp 2</u>		
Virus Isolate	20841	21480	18251	20481	18251
				21480	20481
					21480
<u>9881</u>					
Day 4	5,404*	6,562	2,048	26,249	4,096
Day 7	65,536	32,768	8,192	419,991	14,263
<u>4971</u>					
Day 4	NT	NT	NT	2,048	2,048
Day 7	NT	NT	NT	14,263	5,404

* TCID₅₀ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Kärber.

A second, independent, experiment was performed using pools of these 3 leukopacks to determine the influence of a poorly performing leukopack on a pool of the more sensitive cells. These data show that the addition of 18251, a less sensitive bleed, to a pool of 20481 and 21480 (better individual performers) significantly reduced the titers obtained at days 4 and 7 for the 9881 isolate. The mechanism responsible for this effect is not immediately clear, but initial observations suggest that adherent cell populations appearing on flask surfaces during PHA stimulation of 20481 and 21480 are markedly reduced during stimulation of 18251 and the 3 leukopack pool. These data again point to the importance of pre-screening all donor PBMCs prior to their use in neutralization assays.

Earlier studies in our laboratory have suggested that fewer numbers of cells per well at the start of culture result in better cell growth and higher titers of virus produced. The results in Table 5 summarize those initial studies and demonstrate that for rapid/hi isolates in particular

Table 5

Effect of Cell Density on Cell Proliferation and Virus TCID₅₀

<u>Num of Cells/well</u>	<u>Fold Increase in Cell Numbers</u>			<u>TCID₅₀</u>	
	<u>Virus</u>	<u>Day 4</u>	<u>Day 7</u>	<u>Day 4</u>	<u>Day 7</u>
5 x 10 ⁴	4971.5	8.8	12.8	891	1024
	9881			7131	10809
2 x 10 ⁵	4971.5	2.9	2.9	675	445
	9881			2048	2048

* TCID₅₀ determined in a pre-neutralization virus titration assay and calculated by the method of Spearman-Kärber.

(9881 is but one example) fewer numbers of cells per well may result in significantly improved cell growth and higher early titers of virus. Indeed, prior to using any leukopack or pool of leukopacks these types of analyses will be performed to maximize use of available materials. During the contract period the preneutralization procedure was used to analyze some 25 individual and pooled leukopacks for virus sensitivity against slow/low and rapid/high isolates. Determinations of virus titers were made for no less than 18 international viral expansions and for at least 40 viruses in multiple leukopacks as a prelude to various experimental procedures. This work represented more than 8400 individual cultures of PBMCs.

Several neutralization projects were completed during the first two base years of the contract; these fall into two categories:

1. Testing of vaccinee sera to determine neutralizing potential and specificity, if any, of candidate vaccines for a panel of laboratory virus strains and selected virus isolates, and
2. Screening and titration of naturally-occurring (infected patients) immune sera against viral isolates in an attempt to determine if previously determined genotypes correlate with apparent serotypes.

The general strategy for both categories has been to screen sera against a typical laboratory strain and several clinical isolates; then to carefully characterize the

Figure 3 Neut. Screen-AVEG Panel

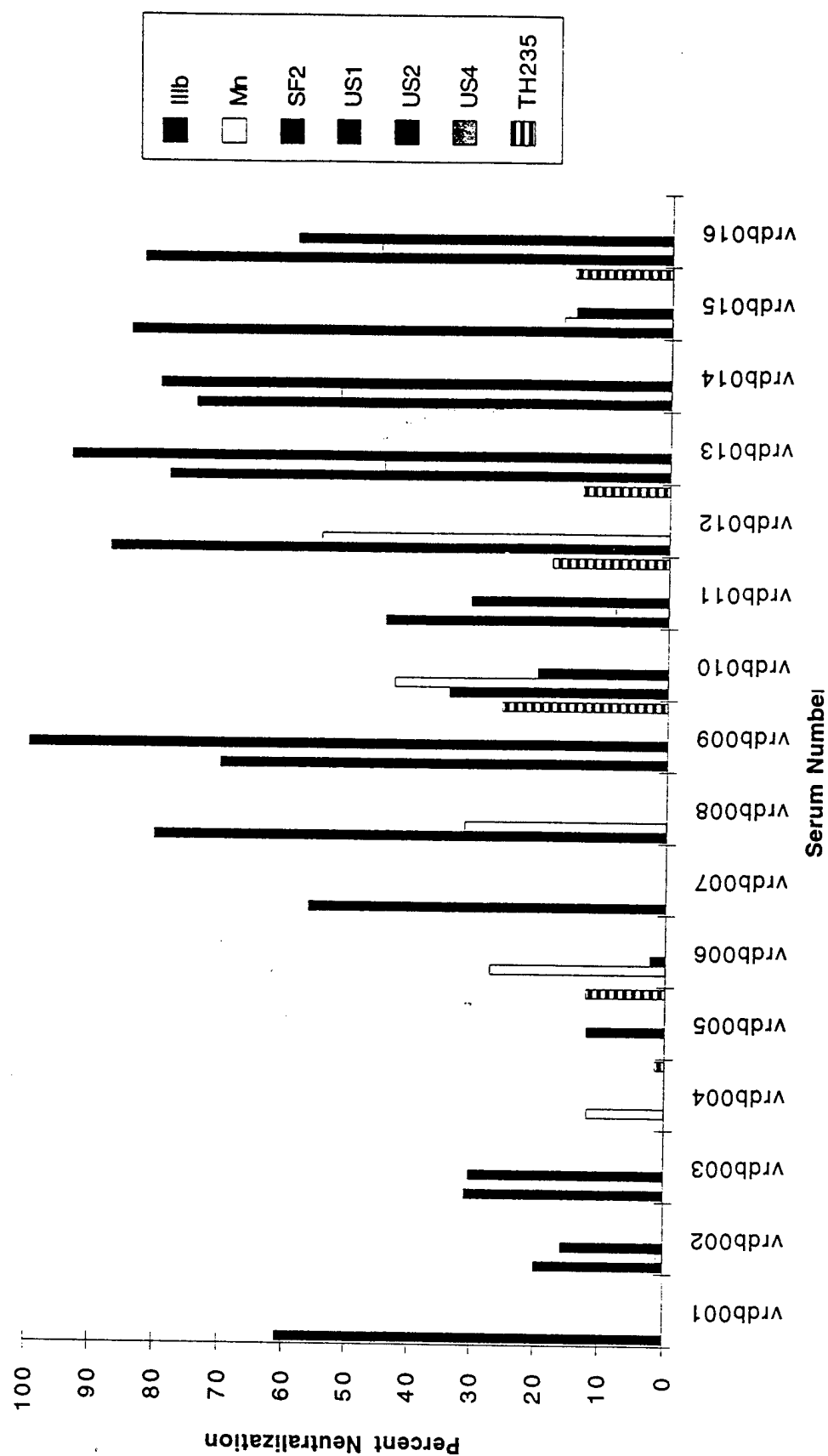


Figure 4 Neut. Screen-AVEG Panel

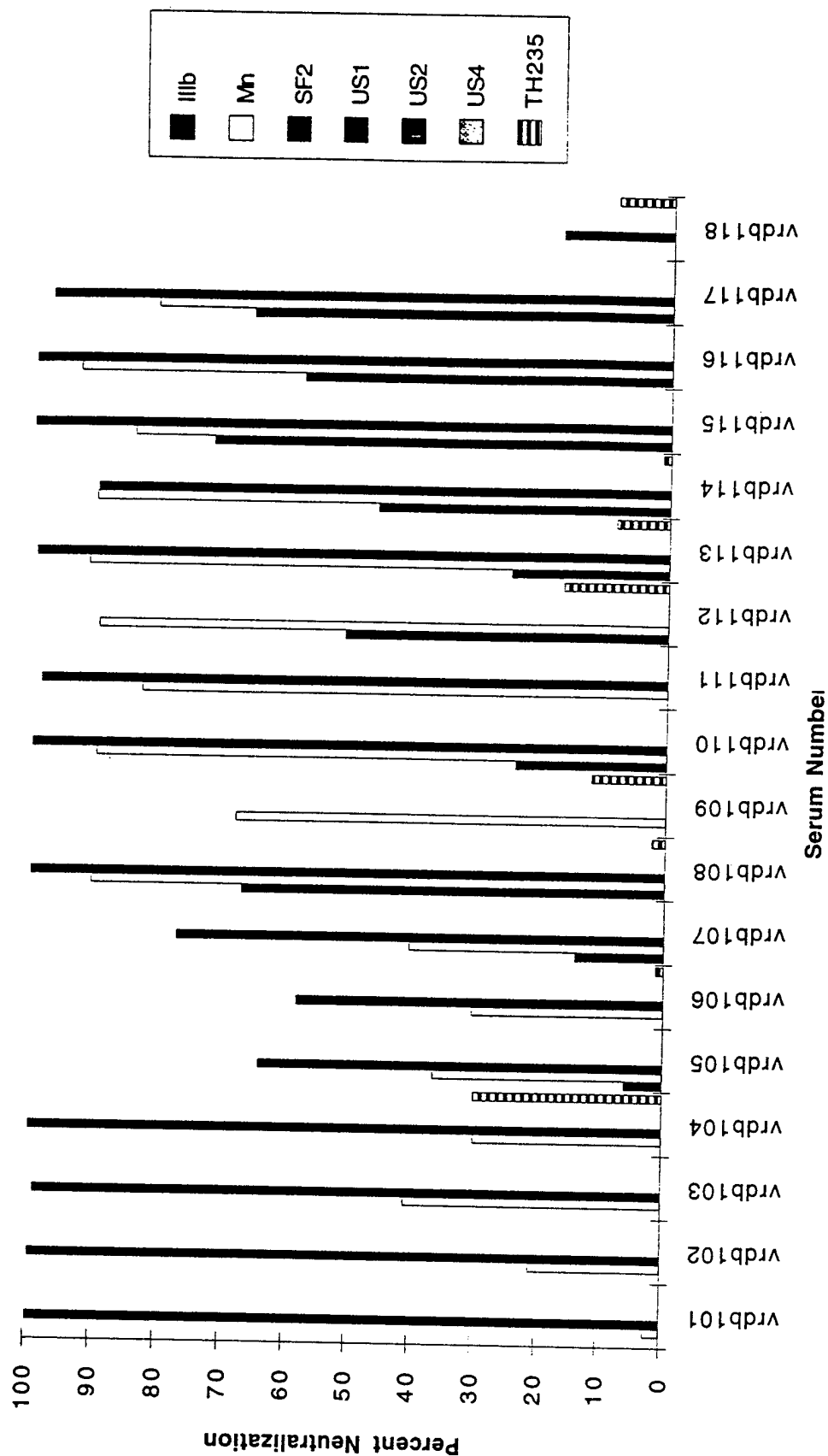


Figure 5 Neut. Screening-AVEG Panel

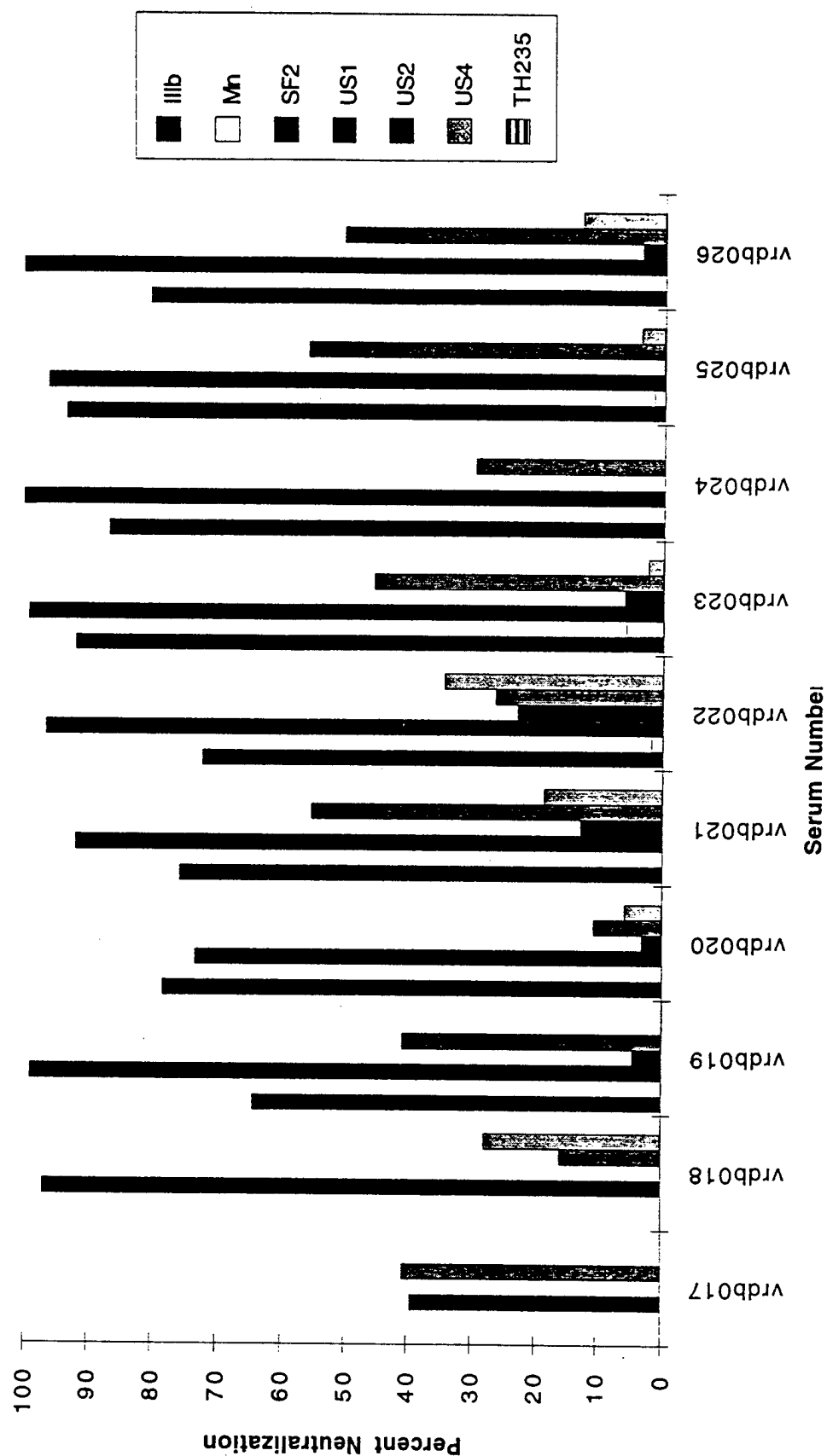


Figure 6 Neut. Screen-AVEG Panel

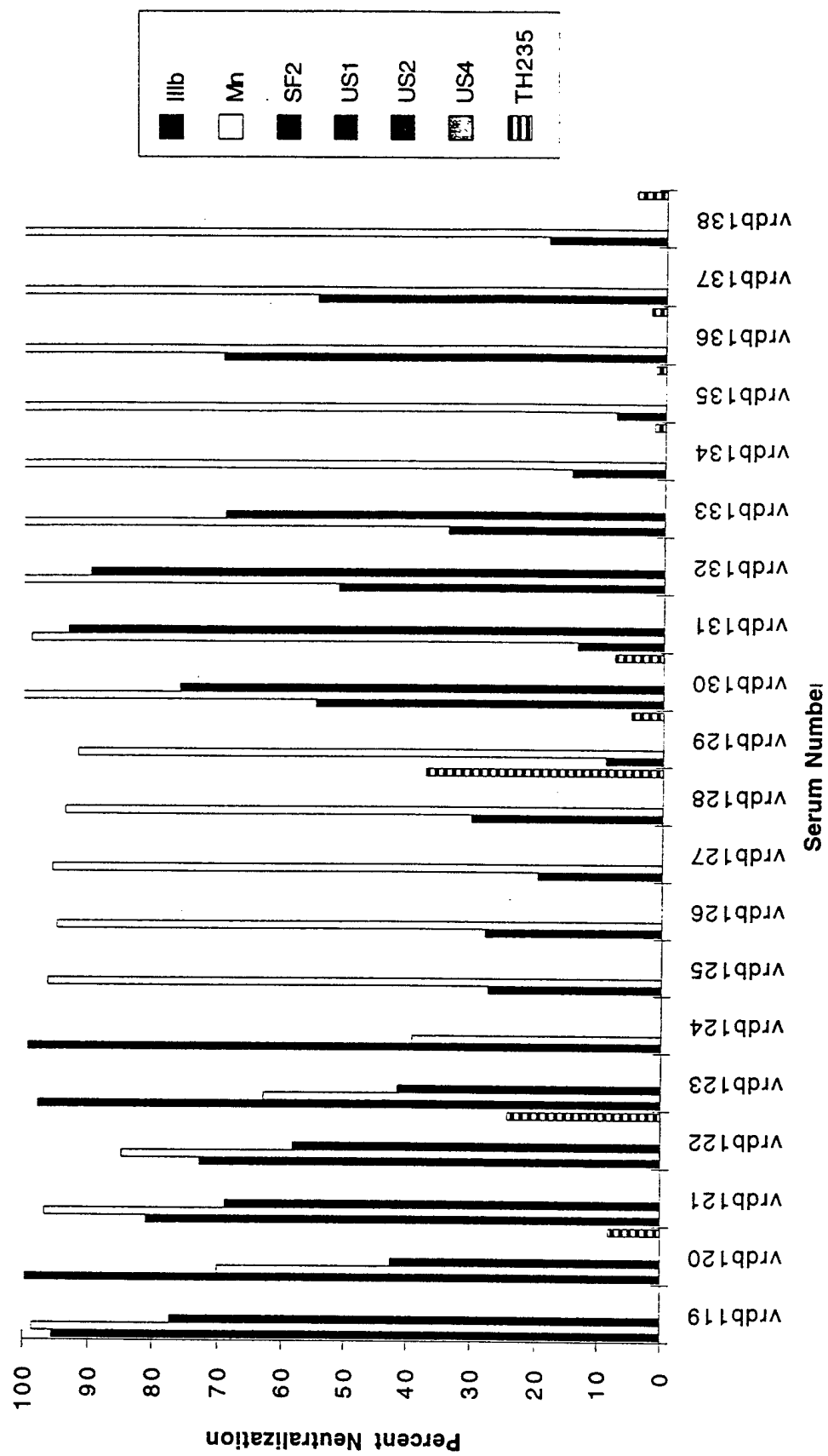


Figure 7 Neut. Screen-lab Strains & Primary Isolates

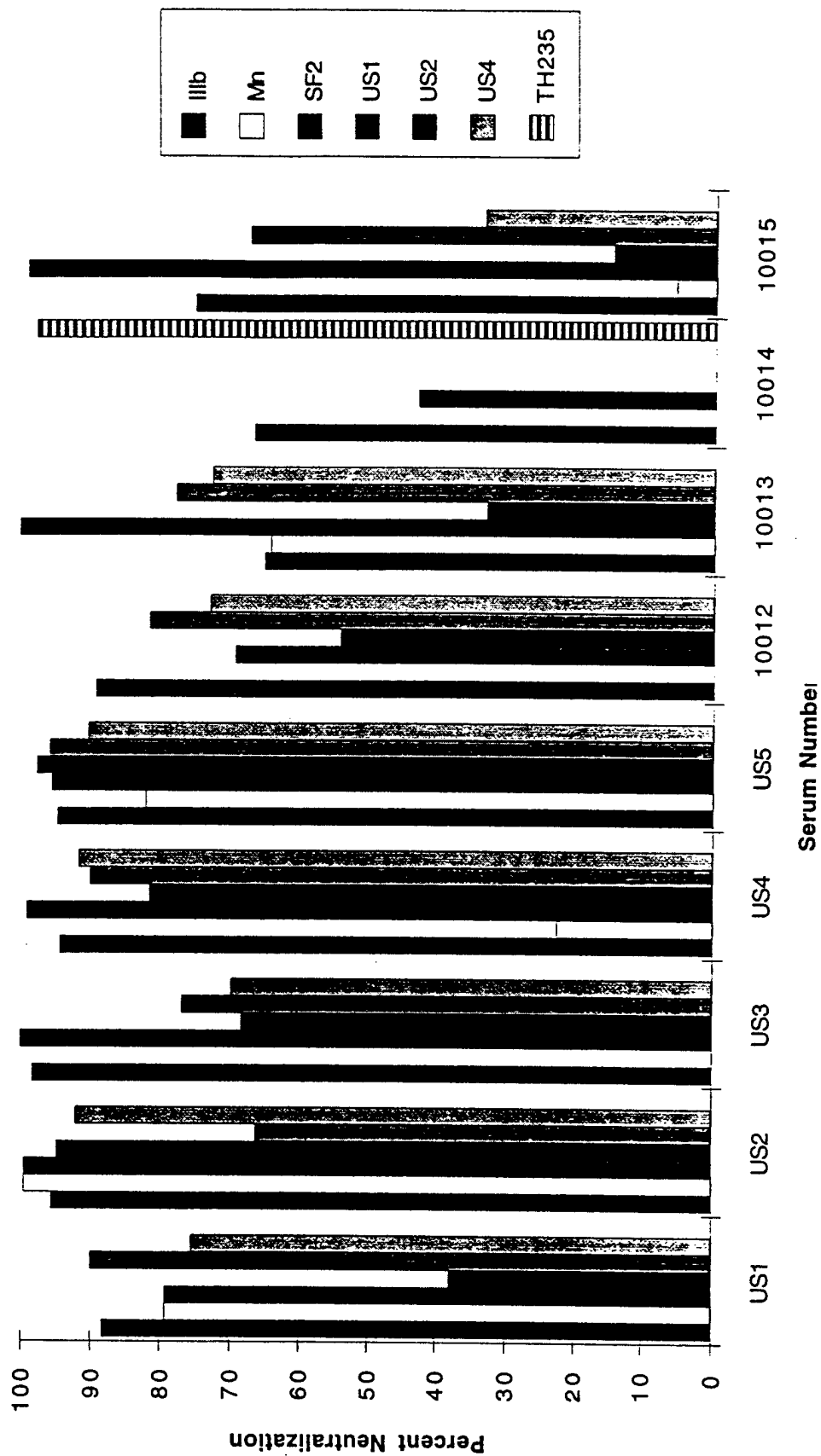


Table 6 Neutralization of Lab Strains of HIV By Patient Sera

SERUM ID	Mn		SF2		IIIb	
	% Neut	Titer	% Neut	Titer	% Neut	Titer
US3	99	14000	99	3500	94	1400
US5	99	26000	99	6200	96	210
Seroneg	<50	<10	<50	<10	<50	<10
VRDB008	<50	<10	<50	<10	86	90
VRDB018	94	110	<50	<10	<50	NT
VRDB103	80	70	90	150	<50	<10
VRDB117	95	12700	96	1500	78	50
VRDB118	<50	<10	<50	<10	<50	<10

* NT: Not Titered

Figure 8 Neut. Screen-International Isolates

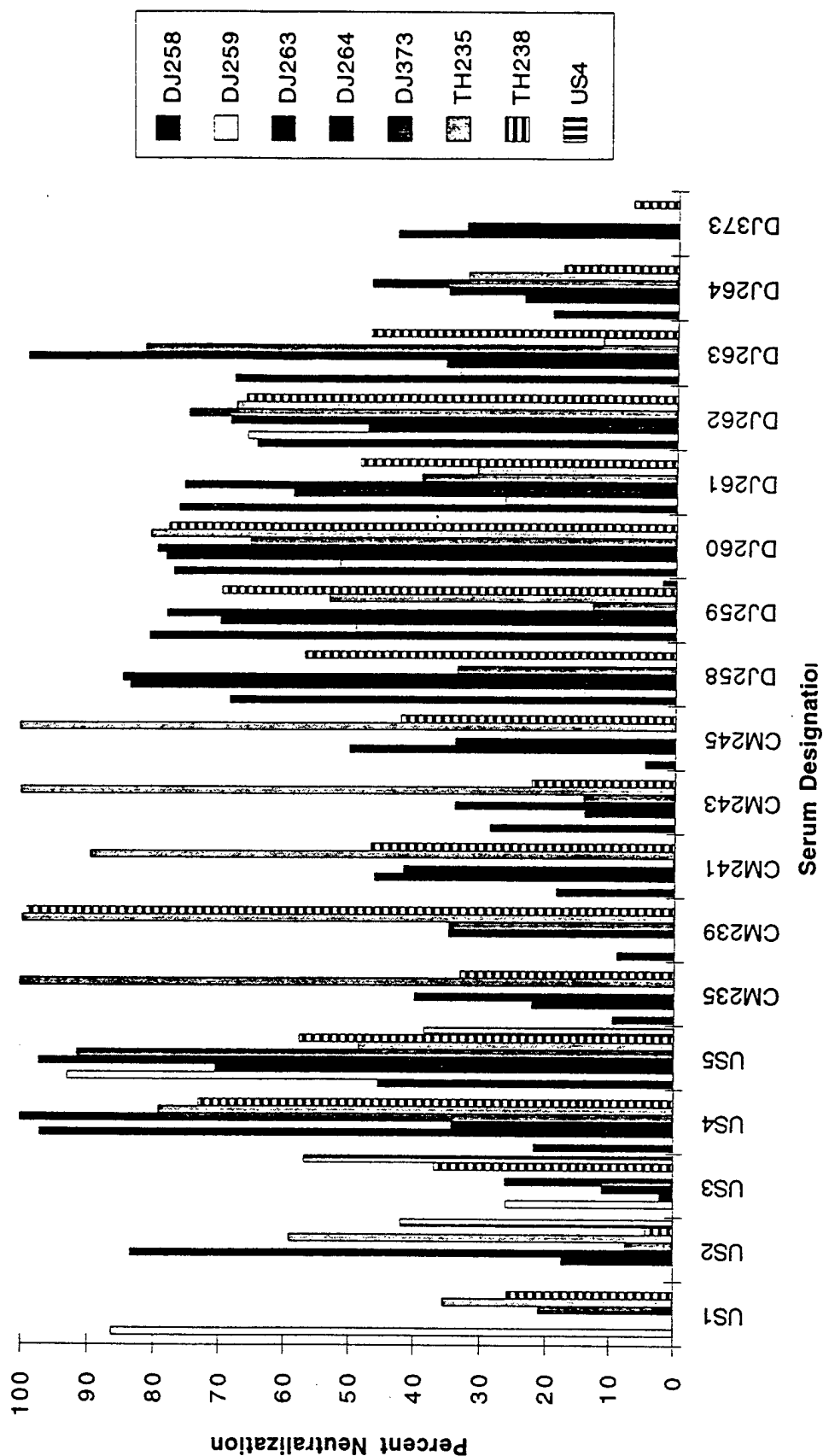


Table 7 SUMMARY OF WHO SERUM SCREENING RESULTS

VIRUS

SERUM	Group A.				Group B				Group C			Group D			Group E		
	RW09		UG37		BR20		TH14		BR25		Mean [p24]	UG24		Mean [p24]	TH22		Mean [p24]
	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index	Mean [p24]	Neut. Index		Mean [p24]	Neut. Index		Mean [p24]	Neut. Index	
RW09	26088	1.50	5418	1.25	24447	2.22	7235	3.19	2858	3.16		318000	2.66		3170	2.77	
UG37	18581	2.11	3038	2.24	8408	6.46	3385	6.83	167	53.98		148914	5.68		1034	8.51	
BR20	11813	3.31	5465	1.24	20743	2.62	5932	3.90	3885	2.32		432200	1.96		2758	3.19	
TH14	21867	1.79	5281	1.29	27202	2.00	7155	3.23	3095	2.92		229200	3.69		4301	2.05	
BR25	25625	1.53	4857	1.40	26348	2.06	6269	3.69	3392	2.66		388800	2.18		3601	2.44	
TH22	14074	2.78	6134	1.11	25680	2.12	11859	1.95	4856	1.86		200781	4.21		3129	2.81	
UG24	23074	1.70	3872	1.75	18243	2.98	3347	6.90	1826	4.94		101886	8.31		1641	5.36	
FDA2	9892	3.96	127	53.55	627	86.59	418	55.28	347	25.99		356400	2.37		1033	8.52	
NHS	39130	1.00	6790	1.00	54324	1.00	23106	1.00	9028	1.00		846200	1.00		8797	1.00	

Table 8 SUMMARY OF WHO VIRUS REDUCTION ASSAY

VIRUS	TCID50 (STANDARD DEVIATION) IN THE PRESENCE OF:							
	NHS(1)		NHS(2)		NHS (mean)		10013	
	TCID50	STDEV	TCID50	STDEV	TCID50	STDEV	TCID50	STDEV
TH235	127	27	143	9	135	6	29	3
US1	1460	43	510	21	808	22	1490	44
							96	33
							9170	3

VIRUS	log[TCID50(NHS)/TCID50(test)]					
	NHS					
	NHS(1)	NHS(2)	(mean)	TH243	US2	
TH235	0.03	-0.03	0.00	0.67	0.15	
US1	-0.26	0.20	0.00	-1.04	0.62	

"neutralizing" sera using serum titration in an attempt to rank the sera. The panels of sera tested in the first category were recieved through Dr. John Mascola from the AIDS Vaccine Evaluation Group (AVEG) and a private biotech firm (Chiron). A summary of the screening from the AVEG can be seen in figures 3-7. The data presented indicate that sera from vaccinees--immunized with glycoproteins from laboratory strains--are able to neutralize laboratory strains, but not primary isolates. The results of the screening were confirmed by further titrating some of the sera that showed significant neutralization, *i.e.*, > 75% neutralization of the virus. Table 6 summarizes representative data from serum titration experiments. In addition to non-neutralizing sera, sera were found that were narrowly neutralizing--*e.g.*, VRDB008 and VRDB018--whereas other sera were broadly neutralizing--*e.g.*, VRDB117.

The sera of the second category were recieved directly from MMCARR collaborators of Dr. Mascola--International Isolates--or from the World Health Organization--WHO. Figure 8 summarizes representative data from the International Isolate panels. In these, as previously reported by Dr. Mascola, sera seem to neutralize better within their genotypic clade.

The experiments using sera recieved from the WHO are part of another effort to serotype HIV. The data of Table 7 represent this laboratory's part in comparing two different typing methods: Serum Titration and Infectivity Reduction Assays--IRA. The data of Table 7 were compared with other data obtained in a collaborating lab (Dr. Peter Nara) and although there is good agreement between the results of the two methods, the IRA results are more amenable to statistical analysis and requires less work; representative data from IRA titrations are presented in Table 8.

Virus Expansions and Evaluation of Cytopathic Effects

A substantial part of this contract's resources were allotted to the expansion and titration of viral stocks. Investigations of the biological characteristics of viral isolates also represents an important part of all clinical protocols supported by this contract. In addition to the existing procedures for virus expansion and titration, SRA established the ACTG's procedure for SI/NSI analysis in house in support WRAIR studies. These protocols can be found in the ACTG Virology Manual and, for that reason, will not be repeated here. The numbers of expansions and evaluations of virus cytopathicity performed by SRA during the three base years of this contract are documented in the appendix.

Neutralization Assay Research and Development

A significant portion of this laboratories support of the HIV interventional trials contract has been Vaccine Development. In collaboration with Dr. J. Mascola, a series of experiments were conducted in the FY/94 contract year to better understand the

results of neutralization assays; we have examined the kinetics of replication of several viruses, the effect and kinetics of neutralization prior to infection, the effect of neutralization concomitant with infection and neutralization after virus binding to cells was thought to occur.

It is well known that different viruses replicate at different rates. We confirmed the many previous observations of others and characterized 4 specific viruses. Characterization of different replication rates is directly relevant to the interpretation of subsequent neutralization experiments; *e.g.*, we need to know if the apparent decrease in replication of the virus in the presence of "neutralizing" antisera is merely an artifact of replication kinetics or true neutralization. The four viruses--two laboratory-adapted strains and two primary isolates--show wide differences in infection rates.

We have also found that laboratory viruses are neutralized more easily (or more quickly) than primary isolates prior to infection. In two separate experiments, virus and antisera were incubated for up to 16 hours prior to the addition of cells. After a standard infection period, the virus and antisera were removed and then viral replication allowed to proceed. The results of each experiment were the same: No statistically significant neutralization of primary isolates by the antisera used was measured in contrast with marked and time dependant neutralization of laboratory-adapted viruses.

In contrast to these results, both laboratory-adapted and primary isolates appear to be neutralized in a time-dependant manner. In several experiments, virus and cells were incubated for up to 16 hours prior to the addition of virus. After a standard "neutralization" period (1-2 h), the virus and antisera were removed and then viral replication allowed to proceed. The results of each experiment were the same: Statistically significant, time dependant neutralization of both primary and laboratory-adapted isolates by the antisera used was measured. The rates of neutralization differed for each virus and did not group according to source; *i.e.*, laboratory isolates are not neutralized faster or slower than primary isolates.

The results described suggest that laboratory-adapted viruses may be neutralized by an additional mechanism to that of primary isolates. The experiments performed to date are preliminary and have certain inherent limitations: Small sample sizes of both virus and antisera, suboptimal experimental control, and--in the earlier experiments--lack of the ability to statistical analyze the data because of experimental design. Using the virus infectivity reduction assay (described above) and newly available statistical evaluation software, we will be able to overcome these limitations. Many of these studies have been published or presented at scientific symposia by Dr. Mascola and scientists at SRA Technologies (see publication and abstract listings at the end of this report).

Evaluation of Antiviral Gene Constructs

Gene therapy for immunological disorders, cancers and a variety of infectious diseases is quickly becoming a reality. This approach has been expanded from "simple" gene replacement or augmentation therapy to correct a genetic defect (as in the case of adenosine deaminase, ADA, deficiency^{36,37} to new genetic treatments for cancers³⁸ and infectious diseases such as AIDS.^{39,40} There have been numerous proposals for the treatment of HIV infections using antisense genes⁴¹⁻⁴⁵ and genes containing catalytic RNAs (ribozymes).^{46,47} *In vitro* interference with viral replication has been accomplished by targeting gene constructs to viral structural proteins⁴⁸⁻⁵², components of HIV's regulatory circuits⁵³⁻⁵⁵ and the virus receptor, CD4.⁵⁶ The number of antiviral gene constructs available for testing appears to be multiplying exponentially.

Preliminary *in vitro* evaluation of these therapies has been accomplished, for the most part, in artificial systems sometimes employing biochemical endpoints or in well established cell lines using laboratory strains of HIV. Little is known about the efficacy of such treatments for primary isolates of HIV in normal human peripheral blood mononuclear cells (PBMC) and there are no published reports of quantitative determinations of putative antiviral gene effects on primary isolate-induced cytopathogenesis. Moreover, the impact of these constructs on the differentiation and ultimate immune function of human bone marrow derived hematopoietic stem cells, the apparent conveyance of choice for some gene constructs, is little understood. Finally, there still is no *in vitro* testing system available to bridge the gap between preclinical *in vitro* analyses and animal model systems such as the SIV model in macques.

In support of a WRAIR's gene therapy research and development, SRA was requested, in the first contract year (FY93) to develop *in vitro* assay systems to assess the efficacy of antiviral gene constructs against low passage, clinical isolates of HIV. Initial studies were to involve the use of syncytial-inducing isolates of HIV in established cell lines previously transfected with antiviral genes. This was to be followed by similar studies in PBMCs that would permit evaluation of a broader range of clinical isolates or, eventually, a prospective patient's own cells. The cell line chosen for the preliminary studies was MT-2, a line that is productively infected with HTLV-1, but is sensitive to infection by 35% of patient isolates. The initial studies conducted with these cells failed in the last fiscal year suggesting that production of HTLV-1 may have blocked the action(s) of the antiviral genes under study. This virus could conceivably interfere with expression, regulation or activities of the antivirals. New studies were conducted in FY94 using the A3.01 and SupT-1 cell lines, both of which are free of HTLV-1. Tables 9, 10, 11 and 12 illustrate the antiviral effects of a number of antiviral gene constructs using both reverse transcriptase and p24 endpoints in SupT-1 or A3.01 cell lines. Spurred by these successes we began to evaluate the possible use of purified CD4+ PBMCs in gene therapy by studying the

Table 9 Effect of Antiviral Gene Constructs on HIV-1 Reverse Transcriptase Production											
Cell Line (SRA #)	pJM #	Description	CD4	Total Cell Count	Cells/Well	% of control growth	RT/Sample	Sample Vol.	RT/Well	RT/10e5 Cells	% Control
Virus = 8119											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	2366667	100.00	473333	180892	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	647667	100.00	129533	52161	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	154618	23.87	30924	16205	31.07
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	56249	8.68	11250	3857	7.39
Virus = RF											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	3475000	100.00	695000	265605	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	536500	100.00	107300	43208	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	664833	123.92	132967	69677	161.26
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	308667	57.53	61733	21166	48.99
Virus = IIIb											
CL094	Supt1	Parental line	+	7.85E+05	2.62E+05	100	2793333	100.00	558667	213503	100.00
CL097	GINA		+	7.45E+05	2.48E+05	95	493333	100.00	98667	39732	100.00
CL098	LRSN3		+	5.73E+05	1.91E+05	73	150840	30.58	30168	15809	39.79
CL099	LRSNDLTA78f		+	8.75E+05	2.92E+05	111	31518	6.39	6304	2161	5.44
CL097 is control for CL098 and 99.											

Table 10 Summary of Antiviral Effects of Gene Constructs in A3.01 Cells

Cell Line (SRA #)	pJM #	CD4	Total Cell Count	Cells/Well	% of control growth	p24/ml	% Control	p24/Well	p24 /10e5 Cells	% Control
490.GT0042	BK89	+	1.24E+06	4.12E+05	94	10	0.00	2	0	0.00
490.GT0043	321neo	-	9.23E+05	3.08E+05	70	1028	0.12	206	67	0.17
490.GT0044	321neo	+	1.52E+06	5.08E+05	116	547167	64.80	109433	21542	55.83
490.GT0045	357B	+	1.16E+06	3.86E+05	88	40909	4.85	8182	2121	5.50
490.GT0047	324	+	8.97E+05	2.99E+05	68	547072	64.79	109414	36593	94.84
490.GT0048	322neo	+	8.28E+05	2.76E+05	63	711000	84.21	142200	51522	133.53
490.GT0049	320neoA	+	1.02E+06	3.40E+05	78	1150148	136.22	230030	67656	175.35
490.GT0050	311	+	1.42E+06	4.72E+05	108	791833	93.78	158367	33529	86.90
490.GT0051	292neoA	+	1.21E+06	4.03E+05	92	497333	58.90	99467	24682	63.97
490.GT0052	160-2	+	1.06E+06	3.53E+05	81	2050000	242.80	410000	116038	300.75
490.GT0053	136	+	1.18E+06	3.94E+05	90	913333	108.17	182667	46323	120.06
490.GT0054	290	+	1.26E+06	4.20E+05	96	646448	76.56	129290	30759	79.72
490.GT0056	188	+	1.11E+06	3.68E+05	84	1171500	138.75	234300	63611	164.87
490.GT0057	253	?	1.21E+06	4.03E+05	92	1111	0.13	222	55	0.14
CL085	322	-	9.49E+05	3.16E+05	72	522	0.06	104	33	0.09
CL086	tat	+	1.05E+06	3.51E+05	80	71	0.01	14	4	0.01
CL087	362A	+	7.67E+05	2.56E+05	58	24118	2.86	4824	1887	4.89
CL088	362B	+	6.24E+05	2.08E+05	48	422	0.05	84	41	0.11
CL089	362A	+	5.72E+05	1.91E+05	44	3188	0.38	638	334	0.87
CL090	354	+	1.04E+06	3.47E+05	79	2426	0.29	485	140	0.36
CL091	tat	+	9.10E+05	3.03E+05	69	384500	45.54	76900	25352	65.71
CL093	A3.01	+	1.31E+06	4.38E+05	100	844333	100.00	168867	38583	100.00

Table 11 Evaluation of Antiviral Gene Constructs

Tech = Louis Davis
Virus = HIV-RF

Cell Lines =		GT77	GT78	TCID50/TREATMENT				GT80	GT81	GT82	GT83	GT84	GT85	GT86
REPLICATES		100	100	100	100	100	100	100	100	100	100	100	100	100
1		71486	952000	1240000	37717	48099	313000	312000	12	670000	294000			
2		60436	821000	187000	78042	29438	1310000	52397	14	855000	122000			
3		72825	1460000	711000	54706	23278	91182	45591	13	955000	486000			
4		62932	888000	149000	50377	138000	152000	34528	13	749000	195000			
5		21542	1370000	544000	27061	116000	209000	88667	13	876000	119000			
No cell control		3015	32005	37819	1227	2374	22536	12472	9	119000	21734			
MEAN		57844	1098200	566200	49581	70963	415036	106637	13	821000	243200			
+/-SD		20981.99	294606.86	445341.11	19266.76	52543.41	506925.07	116581.71	0.71	111939.72	153273.29			
CV		0.36	0.27	0.79	0.39	0.74	1.22	1.09	0.05	0.14	0.63			
% CONTROL*		117	2215	1142	100	9	51	13	0	100	30			

Virus = HIV - 8119

Cell Lines =	GT77	GT78	TCID50/TREATMENT										GT85	GT86
			REPLICATES	100	100	100	100	100	100	100	100	100		
	1	129000	587000	1190000	35618	8552	100000	59421	24	55545	310000			
	2	41688	546000	402000	20254	14598	59654 >>>>>>	22	64976	158000				
	3	20726	761000	315000	45082	22161	276000	172000	20	71446	311000			
	4	203000	579000	475000	40372	32288	331000 >>>>>>	24	78011	650000				
	5	83574	715000	225000	70514	18334	60486	20169	29	67253	544000			
No cell control		11468	17327	41062	1573	411	9264	2294	21	1919	9188			
MEAN		95598	637600	521400	42368	19187	165428	83863	24	67446	394600			
+/-SD		72985.55	94344.05	385321.03	18290.29	8879.34	128571.03	78811.38	3.35	8297.46	198541.68			
CV		0.76	0.15	0.74	0.43	0.46	0.78	0.94	0.14	0.12	0.50			
% CONTROL*		226	1505	1231	100	28	245	124	0	100	585			

Table 12 Evaluation of Antiviral Gene Constructs

Tech = L. Davis
Virus = HIV-1RF

Cell Lines =		GT0096	GT0095	TCID ₅₀ /TREATMENT										GT0088	GT0087
REPLICATES		100	100	100	100	100	100	100	100	100	100	100	100	100	100
1		7951	202000	93966	392000	133000	465000	794000	185000	1168	331				
2		8332	195000	74147	568000	217000	295000	306000	>>>>>>>	>>>>>>>	732				
3		>>>>>>>	270000	19434	53310	138000	99319	190000	257000	871	>>>>>>>				
4		>>>>>>>	216000	64277	>>>>>>>	>>>>>>>	432000	531000	318000	1231	427				
5		>>>>>>>	404000	101000	584000	214000	165000	1160000	81600	827	387				
No cell control															
MEAN		8142	257400	70565	399328	175500	291264	596200	210400	1024	469				
+/-SD		269.41	87073.53	32173.92	246533.40	46249.32	160304.60	390659.95	101626.64	204.78	179.54				
CV		0.03	0.34	0.46	0.62	0.26	0.55	0.66	0.48	0.20	0.38				
% CONTROL*															

Cell Lines =		GT0096	GT0095	TCID ₅₀ /TREATMENT										GT0088	GT0087
REPLICATES		100	100	100	100	100	100	100	100	100	100	100	100	100	100
1		222000	62025	565000	169000	474000	734000	878000	1160000	91	991				
2		57187	156000	201000	224000	501000	477000	839000	651000	538	1972				
3		15103	103000	11392	>>>>>>>	907000	261000	725000	441000	781	>>>>>>>				
4		50129	19831	207000	>>>>>>>	708000	577000	1000000	587000	380	>>>>>>>				
5		186000	113000	40278	239000	646000	1050000	764000	837000	486	>>>>>>>				
No cell control															
MEAN		106084	90771	204934	210667	647200	619800	841200	735200	455	1482				
+/-SD		91682.21	51858.74	220361.85	36855.57	175047.71	295412.76	107283.27	276778.25	251.12	693.67				
CV		0.86	0.57	1.08	0.17	0.27	0.48	0.13	0.38	0.55	0.47				
% CONTROL*															

susceptibility of PBMCs, stimulated with anti-CD28 and anti-CD3 antibodies, to virus infection. Our intention was to use cells, stimulated with these agents, and grown for extended periods *ex vivo*, as vehicles for the transduction of antiviral gene constructs. Our initial evaluation of this approach suggested that cells stimulated in this manner are not susceptible to infection with laboratory or clinical isolates of HIV. The section that follows details our investigations of this phenomenon and describes studies that have led to the discovery of soluble inhibitory factors in the supernatants of CD3/CD28 antibody-stimulated CD4+ T-cells.

Identification of Putative Anti-HIV factors in Long Term Cultures of CD4+ T-Cells Intended as Targets for Anti-Viral Genetic Therapy

A process for the *in vitro* expansion of CD4+ T-lymphocytes obtained by apheresis of normal donors or HIV-infected patients has been developed in Dr. Carl June's laboratory that utilizes partially purified PBMCs or purified CD4 cells co-stimulated with antibodies to CD3 (T3) and CD28 (9.3) covalently linked to paramagnetic microbeads. The cell population resulting from this stimulation is CD4+ with no apparent contaminating monocytes, macrophages or CD8 cells. Peripheral mononuclear cells from nine HIV-infected patients have been cultured to date and maintained thru multiple generations, some in excess of 100 days. Cell numbers have grown from 10^5 at the start of culture to, in some cases, well over 10^{12} at culture termination.

These cells are intended as targets for ex-vivo modification by antiviral therapeutic genes or drugs followed by re-infusion into their autologous hosts. An important component of these investigations is the pre-infusion testing of antiviral gene efficacy *in vitro*. Most efficacy analyses of antiviral gene constructs are conducted in established cell lines with laboratory adapted strains of HIV. Evaluation of antiviral therapies with clinical isolates in established cell lines is limited to but 35% of isolates (syncytial inducing), usually obtained from individuals late in their course of disease. Effective determination of efficacy utilizing primary isolates of HIV in normal donor PBMC has been difficult because of the short term nature of PBMC cultures and problems arising from the efficiency of transduction and selection of target cells. The effective longterm culture of PBMCs from normal donor and HIV-infected patients by combined a-CD3/a-CD28 (T3/9.3) stimulation obviates the first of these problems, but treatment efficacy analysis requires these cells (when employed as no treatment controls) to be sensitive to virus infection. Previous studies indicated that stimulation of PBMCs with soluble T3/9.3 antibodies results in the activation of latent HIV infection (3) in patient cells and predicts that normal donor T-cell sensitivity to HIV infection should result from such stimulation. The present investigation was initiated, in part, to develop a system for the efficacy analysis of antiviral gene constructs produced by WRAIR scientists and, in part, to confirm T3/9.3-induced, CD4+ T-cell, sensitivity to HIV infection. We report here the rather

surprising result that T3/9.3 solid phase (in cis) stimulation yields a cell population that is reversibly resistant to HIV infection or transmission and actively transmits this suppression, by way of a soluble factor(s), to cells normally susceptible to infection.

To evaluate the susceptibility of long term PBMC cultures to HIV we attempted to infect normal donor cells, stimulated with T3/9.3 (cis) microbeads, with a US (clade B) clinical isolate of HIV-1 (9881) isolated, expanded and titrated in PBMCs. Normal donor PBMCs were stimulated for three days with either PHA or T3/9.3 covalently linked to paramagnetic microbeads and compared for sensitivity to HIV infection. The results of this study (Table 13) suggested that T3/9.3 stimulation prevented or depressed HIV infection while cells from the same donor, stimulated with PHA, were fully susceptible to infection. No virus p24 production was detected in culture supernatants derived from T3/9.3 stimulated cells through day 21 in the presence of the T3/9.3 microbeads, while PHA-stimulated cells produced 18,820 pg of p24 by day 7 and more than 65,000 pg by day 14. Microbeads, lacking T3/9.3 antibodies were not inhibitory in this study (data not shown). Subsequent investigations (data not shown) showed that the T3/9.3 stimulated cells were not devoid of the CD4 receptor for HIV and HIV infection of these cells did not depend on an altered requirement for polybrene pretreatment.

Several questions are raised regarding this inhibition including: 1) whether the microbeads in some way interfere directly with infection, 2) whether inhibition is unique to the leukopack or virus in question or 3) to the method of stimulation, 4) to the mechanism of inhibition, 5) to the cell populations involved and finally, 6) whether inhibition of HIV infection is reversible. To address these issues we performed a series of studies with additional leukopack-derived cells from different normal donors. Ficoll purified PBMCs were stimulated with T3/9.3 on microbeads. After three days the microbeads were removed from half the cells by centrifugation on ficoll gradients, portions of these bead-free cells were either reincubated or stimulated with PHA. Portions of the remaining, bead-free "resting" cells were similarly stimulated with PHA on days 6 and 10. All PHA stimulated cells along with their unstimulated and T3/9.3 antibody bead containing controls and were infected with 100 TCID₅₀s of HIV-19881 and evaluated seven days later for the presence of culture supernatant p24. These "rested" cells were then restimulated, but with PHA alone, and three days later compared with T3/9.3 stimulated cells (with beads present throughout) for sensitivity to 100 TCID₅₀s of HIV-1₉₈₈₁. T3/9.3 stimulated cells, with beads present or from which antibody containing beads had been removed produced no detectable or barely detectable supernatant p24 throughout the 10 day experimental period (Table 14). PHA stimulation of T3/9.3 stimulated, bead-free cells produced no detectable supernatant p24 until they rested for at least 6 days. These results suggest that suppression of HIV infection by stimulation with antibodies to T3/9.3 anchored on paramagnetic microbeads is reversible and offers the

Table 13 HIV-1 Virus Titrations in CD3/CD2 and PHA Stimulated PBMCs			
<i>Cell Group</i>	<i>Day 7</i>	<i>TCID50</i>	<i>Day 21</i>
		<i>Day 14</i>	
CD3/CD28 Sti	<64*	<64	<64
PHA Stim.	18820	>65000	>65000

Cells infected with US 1 Isolate

Culture supernatants evaluated for p24 on the indicated days

*TCID50 Determined by the method of Spearman-Kärber

Table 14 Effect of CD3/CD28 Mitogen Removal on Sensitivity of Patient Cells to HIV Infection				
<i>Cell Group</i>	<i>Description</i>	<i>Day of Mitogen Removal</i>		
		3	6	10
1	734714 , CD3	135	64	55
2	737414, Beads	90	39	64
3	734714, PHA	<1000	>50000	>250000

Table 15 Virus Titrations in PHA and CD3/CD28 Stimulated PBMCs With or Without Monocyte/Macrophages			
<i>Cell Group</i>	<i>US1 TCID50</i>	<i>Cell Group</i>	<i>BAL TCID50</i>
	<i>Day 7</i>		<i>Day 7</i>
PBMC+PHA	332555	PBMC+PHA	241029
OKT3/9.3 Bea	279	OKT3/9.3 Bea	77
CD4,M/M+PHA	>390625	CD4,M/M+PHA	>390625
CD4,M/M+Bea	386	CD4,M/M+Bea	279
CD4 + Beads	1012	CD4 + Beads	386
SRA PBMC Cor	241029	SRA PBMC Cor	174693

Culture supernatants evaluated for p24 on the indicated days

TCID50 Determined by the method of Spearman-Kärber

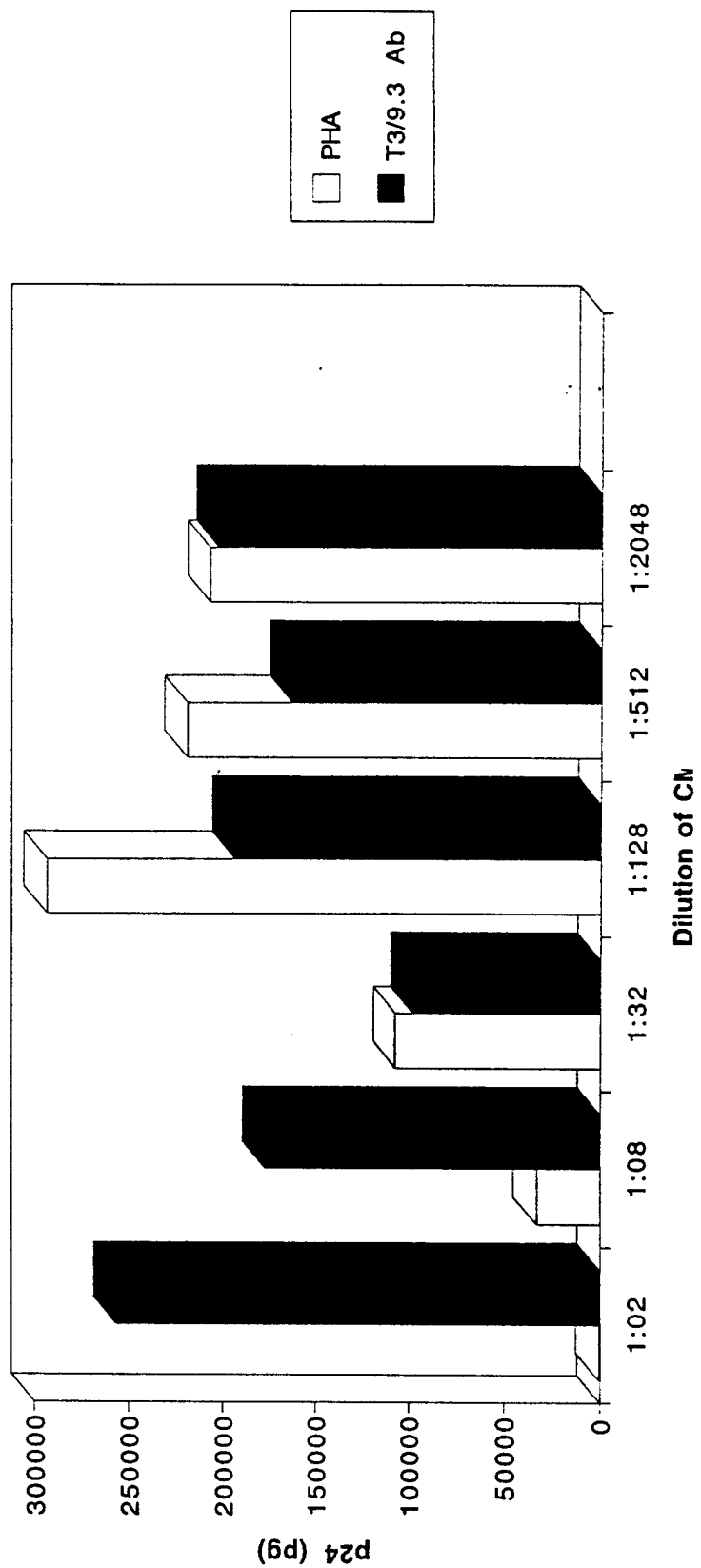
Table 16
Effect of T3/9.3 Stimulated Cells on Virus Infection in PHA Stimulated Cells

Cell Group	Number of Cells		p24 with PHA PBMC: no added CD3/CD28 Cells	p24 with PHA PBMC: + added CD3/CD28 Cells	% of PBMC Control p24
	CD3/CD28	PHA PBMC			
1	0	200000	68063	-	100
2	20000	160000	93076	2193	2
3	40000	120000	96131	1617	2
4	80000	80000	77666	521	1
5	120000	40000	48404	234	0
6	160000	20000	25564	75	0
7	200000	0	-	194	-

Table 17 EFFECT OF CONDITIONED MEDIA FROM T3/9.3-STIMULATED CD4 CELLS ON HIV-1 INFECTION IN PHA-STIMULATED PBMCs				
CELL GROUP	Supernatant p24*	% Inhibition	=/-SD	CV
PBMCs/PHA+IL2	16373		14996	0.916
PBMCs/PHA+IL2+50%CM(PHA Stim)	41080	0	35731	0.870
PBMCs/PHA+IL2+50%CM(T3/9.3 Stim)	1285	92	716	0.557

Table 18 Effect of Conditioned Media From CD3/CD28 Stimulated Cells on Sensitivity to HIV Infection		
Cell Group	TCID50	Percent Reduction
PBMCs+PHA (Autologous Donor)	91767	--
PBMCs+PHA (Heterologous donor)	66511	--
PBMCs+Fresh Cond. Autologous Sup.	3671	96
PBMCs+Frozen Cond. Autologous Sup.	18353	80
PBMCs+Fresh Cond. Heterologous Sup.	18353	72

Figure 9 Conditioned Media Titration



possibility of establishing long-term cultures and large repositories of CD4+ cells from individual normal donors. This would reduce assay-to-assay variability and enhance reproducibility for many HIV test systems. In similar studies, infection with HIV-1_{BAL}, a monocytotropic variant, was also inhibited in PBMC or CD4+ T-cell cultures reinforced with 10% monocyte/macrophages and stimulated with T3/9.3 microbeads (Table 15). Studies with other clinical isolates of HIV-1 are underway to confirm the general applicability of this phenomenon.

A series of experiments were directed toward determining whether inhibition of infectivity by T3/9.3 results from active suppression by stimulated cells or their lymphokines. Purified CD4+ T-cells were washed free of T3/9.3 linked microbeads after 3-4 days of stimulation. Dilutions of these cells were then mixed with PHA-stimulated PBMCs and the cocultures infected with HIV-1₉₈₈₁ and evaluated seven days later for supernatant p24 as described in the methods. PHA-stimulated PBMCs alone produced 68,000 pg of p24/ml of supernatant while T3/9.3-stimulated cells alone produced only 194 pg/ml in seven days. HIV p24 synthesis was inhibited 98% in cocultures containing as little as 10% T3/9.3 - stimulated CD4+ cells (Table 16).

Subsequent experiments have suggested that this inhibition is the result of a soluble factor(s) present in supernatants derived from the T3/9.3 - stimulated CD4+ cell cultures. Conditioned media, derived from either T3/9.3 - stimulate, but not PHA-stimulated PBMCs or CD4+ cells 3-4 days following stimulation suppressed production of supernatant p24 in PHA-stimulated PBMCs or CD4+ target cells (Table 17). Additional studies, employing 50% conditioned media suggested a marked inhibition of HIV infection in both autologous and heterologous cells (Table 18). Frozen supernatants were capable of inducing inhibition in PHA stimulated cells from both autologous and heterologous cells, but to a somewhat lessor extent when compared with fresh autologous supernatants. Figure 9 illustrates the results of a titration study comparing conditioned media from PHA- and T3/9.3 stimulated cultures. The data suggest that a factor or factors, present in conditioned media obtained from T3/9.3-, but not PHA-stimulated cells inhibits the infection, transmission or expression of HIV in a dose dependent fashion. Efforts to establish a CRADA with the Naval Medical Research Institute and Dr. June were unsuccessful. Continued interaction between Dr. June's laboratory and SRA did continue, however, and resulted in the identification of antiviral factors directed toward "T-tropic" SI variants of HIV-1 in established cell lines and PBMCs. The data supporting these ongoing studies are reviewed below.

To review briefly, co-stimulating CD4+ cells with antibodies to CD3 and CD28 conjugated to paramagnetic microspheres enhances production of the chemikines Rantes, MIP-1 and MIP-1, downregulates the HIV-1 co-receptor CCR5, but not CXCR4 and inhibits infection by "M-tropic" or NSI isolates of HIV-1. Infection of these cells by SI isolates (CXCR4 requiring) is not impeded. However, we determined that conditioned medium supernatants from cultures of these cells block infection of

PHA-stimulated PBMCs by both NSI and SI isolates and established cell lines by SI isolates. These latter studies are discussed further below.

We noted above that stimulation of peripheral blood mononuclear cells or purified CD4 T-cells, by antibodies to CD3 and CD28 covalently linked to paramagnetic microbeads, results in the long term proliferation of these cells and suppression of HIV infection in the absence of antiviral drugs. There are at least two components to the HIV inhibitory effects of CD3/CD28 antibody costimulation. An extracellular, soluble component and a cell associated component. The cell associated component apparently involves the down regulation of the HIV co-receptor CCR5, but not CXCR4. The soluble component(s) have been identified as chemokines Rantes, MIP-1 and MIP-1 and their interaction with CD3/CD28 antibody microbead treated cells results in resistance to infection with "M-tropic" NSI variants. Such cells retain their sensitivity to infection with "T-tropic" SI variants. This inhibitory activity is transferable with conditioned medium (CM) from cultures of the bead treated cells. The earlier cells are exposed to CM, relative to infection, the greater the inhibitory activity. Though not shown, conditioned Media sups from PHA-stimulated CD4-cells are not inhibitory.

I should note that studies performed by Dr. June's laboratory, in collaboration with Dr. Berger, have suggested that this entry block results from the failure of the virion's envelope to fuse with the target cell's plasma membrane.

Recently we've begun studies of conditioned media to determine the range of its antiviral activity. What we discovered to our surprise, and I might add, to our confusion, was that conditioned medium from CD3/CD28 antibody co-stimulated cells also inhibits the replication of "T-cell tropic" SI isolates in PHA-stimulated PBMCs and established cell lines.

Figure 10 shows the effect, on HIV-1_{RF} infection, of treating the established T-cell line MT-2 with AZT, CM or a mixture of C-C Chemokines. AZT markedly inhibited RF infection in this cell line while the mixture of C-C Chemokines had no effect. The latter result was, of course, not unexpected, since RF, a "T-cell tropic" variant should employ the CXCR4 co-receptor for entry. However, to our surprise, the conditioned medium supernatant, from CD3/CD28 antibody co-stimulated cultures, had significant antiviral activity. Recall that the cells producing this supernatant are sensitive to infection with "T-cell Tropic" viruses. We've obtained similar results with HIV-1_{IIIb} in MT-2 cells and with both RF and IIIb viruses in CEM-SS cells. We have found that CM will block replication of SI isolates and NSI isolates in both PBMCs and established cell lines while chemokine mixtures will not (data not shown).

Figure 10
Effect of AZT, CM and Chemokine Mixture on HIV-1_{RF} Infection of MT-2 Cells

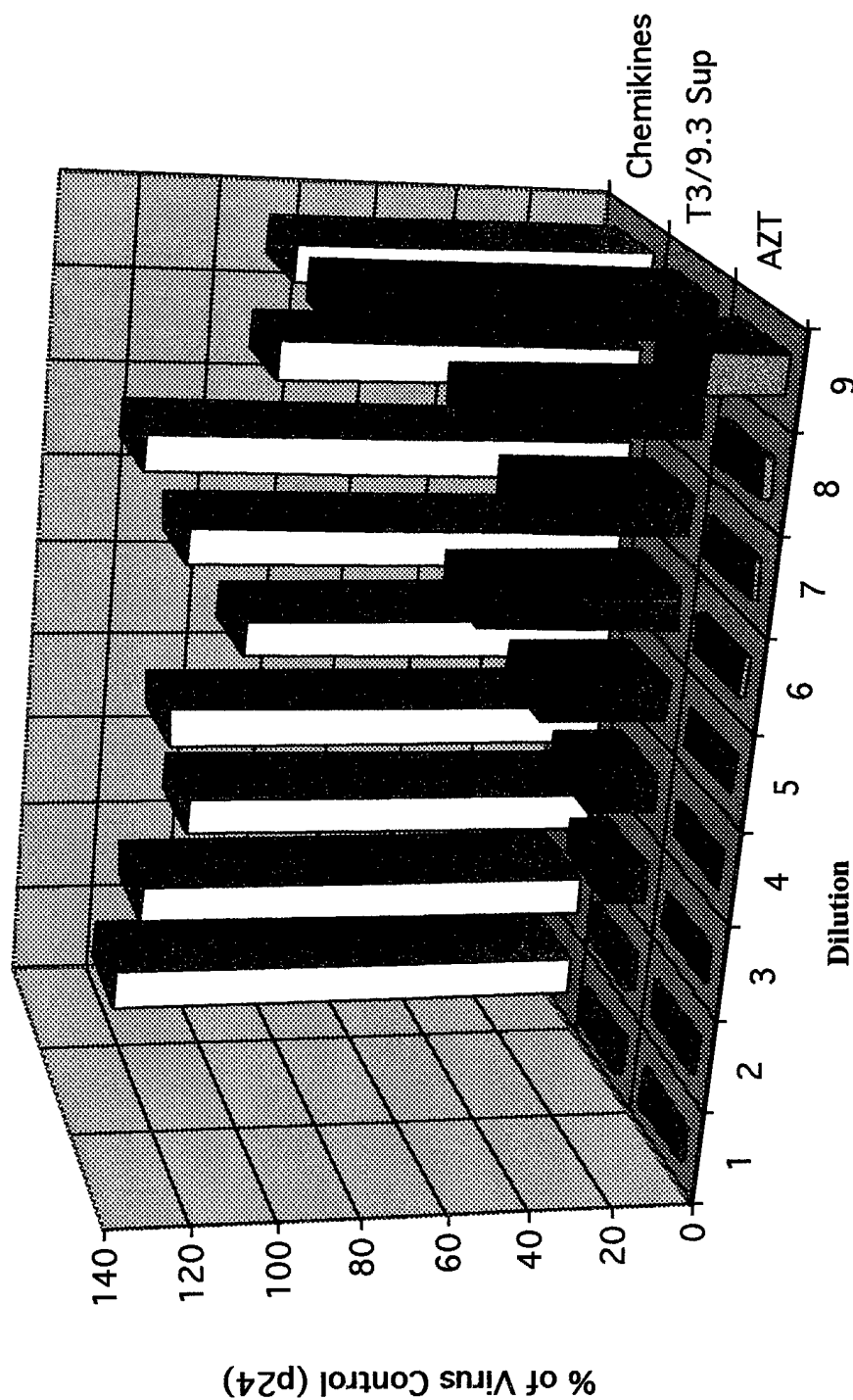
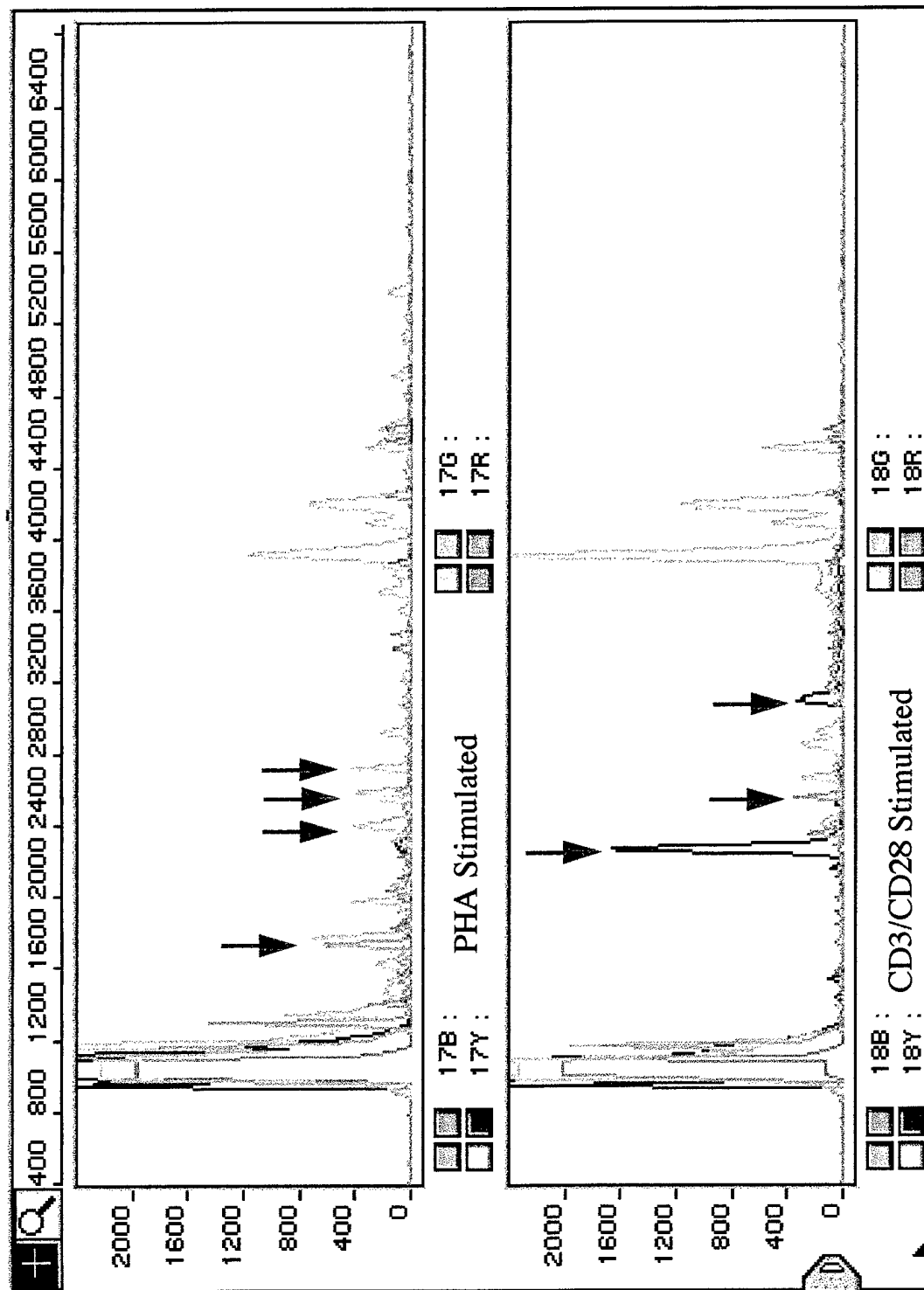


Figure 11



To reiterate, antibody co-stimulation results in a target cell that is resistant to "M-tropic" NSI viruses, but sensitive to infection with "T-tropic" SI viruses. Conditioned medium supernatants derived from cultures of these same cells, inhibit infection by both viral subtypes in PHA-stimulated CD4 cells. This same CM also inhibits "T-cell tropic" or SI virus infection of established T-cell lines. At present we have no explanation for these seemingly conflicting results. We've begun to analyze the supernatants for the presence of CXCR4 ligands, antagonists and other factors.

In addition to evaluating components of the conditioned medium supernatants, we have begun to fingerprint CD3/CD28 co-stimulated cells, PHA-stimulated cells and conditioned medium-treated cells for differentially expressed genes using a newly developed rapid Fluorescence-Based Differential mRNA Display System developed at SRA Life Sciences⁵⁷ in hopes of resolving these conflicting data. These data are revealed in Figure 11. This electropherogram illustrates the differential expression of genes in cells stimulated with either PHA (the top panel) or CD3/CD28 antibody coated beads (the bottom panel). Obtained from a modified sequencing procedure on the ABI 377 automated sequencer, this gene expression discovery system (GEDSSM) suggests that multiple gene expressions occur in one condition and not the other. We are in the process of isolating and identifying these expressed genes in the hope that this approach will provide some insight as to the mechanisms involved.

As for the mechanism of the CM antiviral effect in established cell lines, we've determined that inhibition of virion fusion with target cell membranes is probably not a contributing factor for the suppression of T-tropic virus infections. Graham Allaway of Progenics Pharmaceuticals, using his proprietary resonance energy transfer-based fusion assay, has determined that these supernatants, active against both the "M-tropic" and "T-tropic" subtypes, markedly inhibit CCR5, but not CXCR4-based fusion events.

As noted above, inhibition of HIV infection by CD3/CD28 antibody co-stimulation, or by the conditioned medium supernatants produced by cultures of cells stimulated in this fashion, occurs in the absence of antiretroviral drugs. To date, there is little information available on possible interactions between the current crop of clinically approved antiviral drugs and chemokines, their analogs or chemokine receptor antagonists. However, since the mechanisms of chemokine-induced antiviral activity are unique, it would be logical to expect some synergy with other anti-HIV drugs. We have begun to investigate this question using conditioned medium against "T-tropic", SI isolates in established cell lines.

Figure 12 graphically illustrates the results of a drug combination experiment with Indinavir (MK639) and Conditioned medium (CM) harvested from CD3/CD28 antibody co-stimulated CD4 cells. The data for this study was obtained from a series of 5 microplates containing the replicate drug matrices. The assay was conducted in MT-2 cells, used the SI "T-tropic" laboratory strain HIV-1_{RF} as target and employed the metabolic reduction of the tetrazolium salt XTT to a colored formazan as the endpoint. The raw data was analyzed by

Figure 12

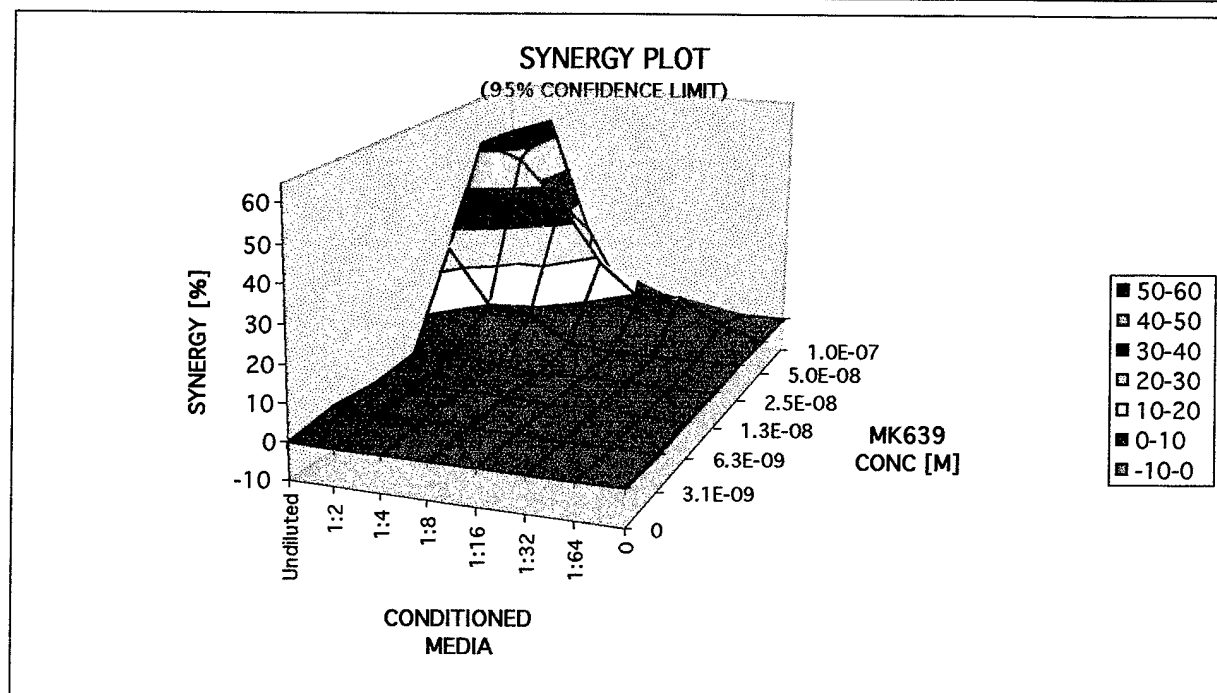
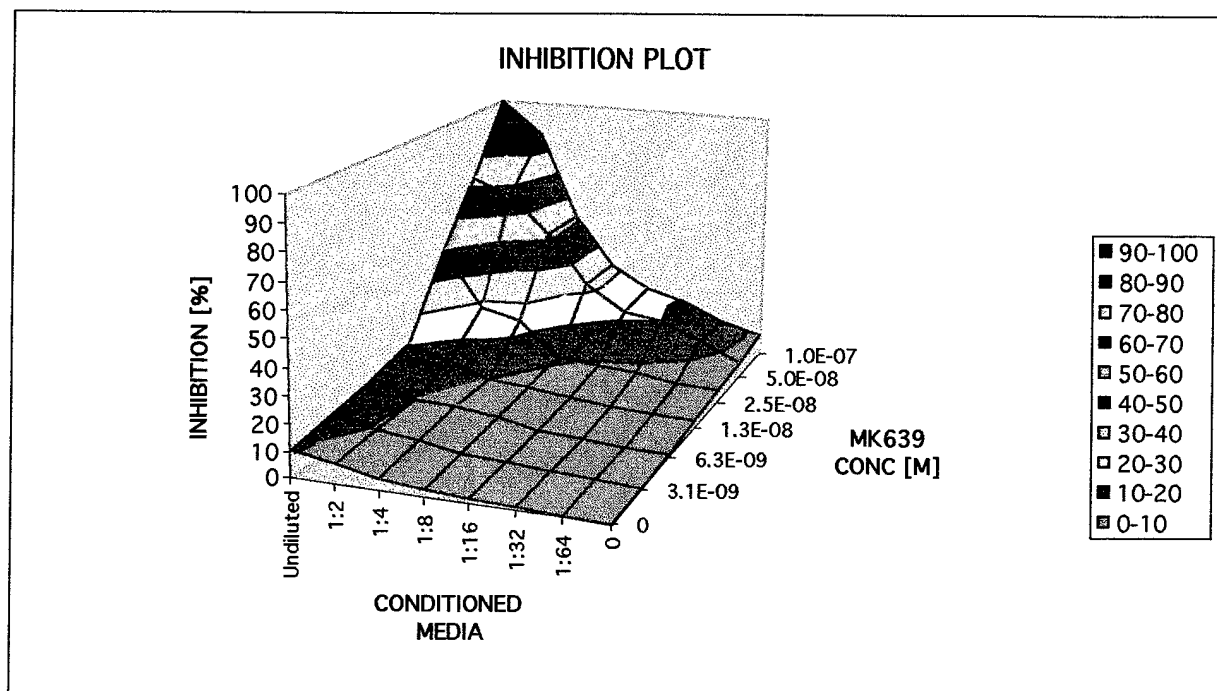
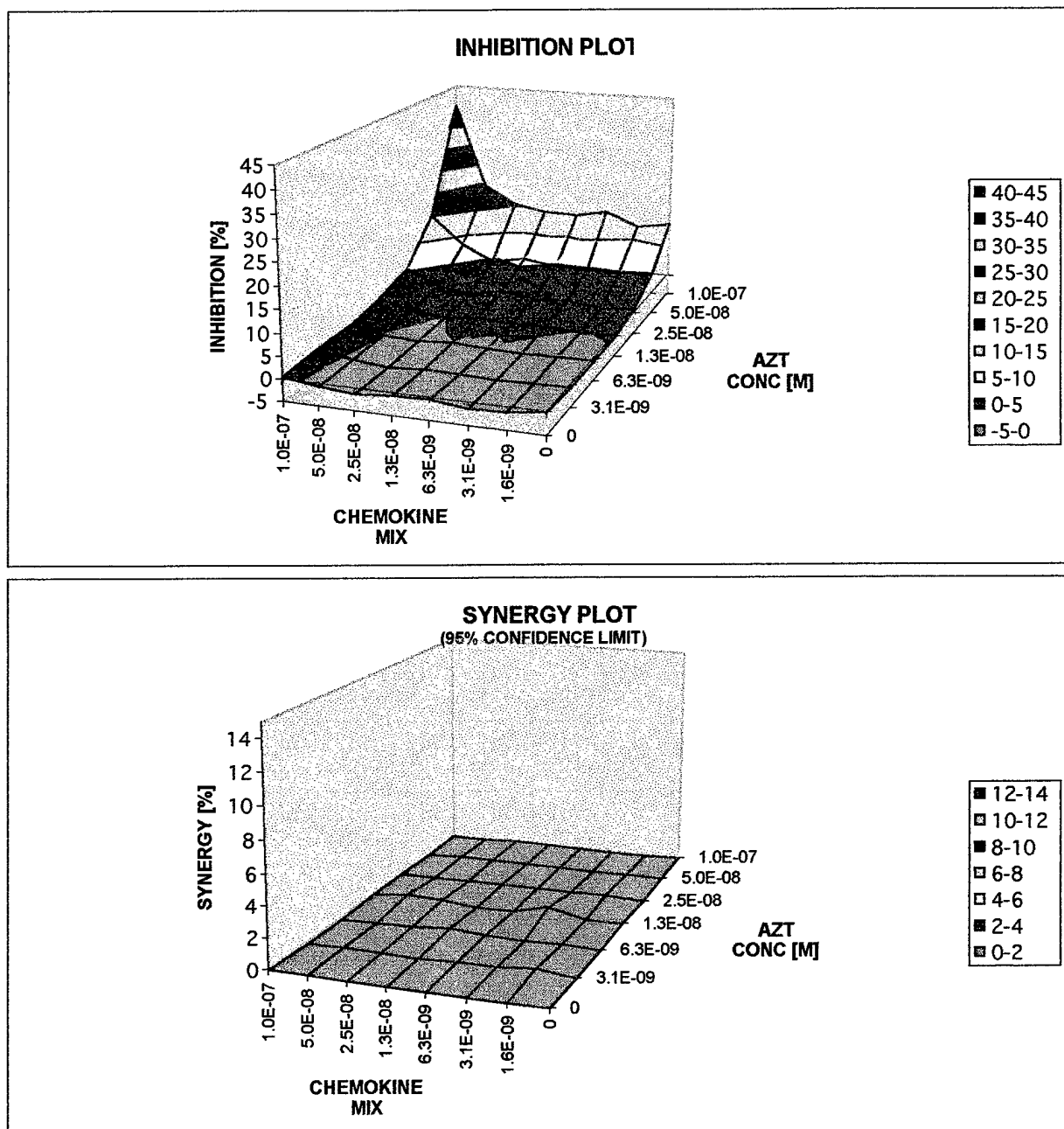


Figure 13



the MacSynergy software package of Prichard and Shipman for two drug combinations and the calculated three dimensional dose-response surfaces ported to Excel for graphical analysis, as shown here, to help in identifying regions of synergy or antagonism.

Theoretical additive interactions are calculated from the dose response curves for each drug used individually. This calculated additive surface, which represents predicted or additive interactions is then subtracted from the experimentally determined dose-response surface to reveal regions of non-additive activity. That is, antagonism or synergy. The resulting surface appears as a horizontal plane at 0% if the interactions were merely additive. Any peaks above this plane would be indicative of synergy. Similarly, any depression in the plane would indicate antagonism.

Prichard and Shipman have established guidelines for determining the significance of synergy or antagonism volumes based on their experience in analyzing antiviral drug combinations. At the 95% confidence interval, values of synergy or antagonism under 25% are regarded as insignificant and probably not important. Values between 25% and 50% are considered to represent minor, but significant synergy or antagonism. Values of 50%-100% indicate moderate synergy or antagonism that may be important in vivo; and values over 100% are indicative of strong synergy and are probably important in vivo. In this case, the combination of MK639 and CM results in moderate synergy. The specificity of these results, or rather the restriction to SI or "T-tropic" viruses in this case is confirmed by the failure of a chemokine mixture to 1) block infection with HIV-1_{RF} or potentiate the antiviral activity of conditioned media (Figure 13).

These studies, initiated at the request of the DoD will be continued following the contract's termination at SRA's expense, in collaboration with Dr. June's laboratory.

3. Antiviral Drug Testing Group

The principal focus of the antiviral drug testing group was the utilization of existing *in vitro*, peripheral blood mononuclear assays to determine drug susceptibility of patient isolates in support of WRAIR clinical protocols. In addition, this unit performed phenotypic analyses for drug resistance and has developed, in collaboration with contract CORs a rapid screening assay for identification of drug resistance at the time of virus isolation. The protocol for this is described below. Finally, the drug testing group had the responsibility to evaluate putative antiviral agents developed by WRAIR scientists. During the period 10/1/92-9/29/97 the drug testing group supported the following studies:

1. RV43 - to determine the incidence and clinical significance of AZT resistance in patients with HIV disease being treated with AZT.
2. RV65 - to determine the time course of development of resistance to an experimental compound (here called compound A) in patients with HIV isolates demonstrating *in vitro*

resistance to AZT.

3. RV79 - An ACTG/NIAID sponsored clinical evaluation of the codon 215 genotypic assay. To date more than a hundred specimens have been evaluated for the presence or absence of this phenotype. A substudy evaluating viral burdens and genotype was added to this protocol in FY96/97.
4. CPCRA (007/014) - prospective evaluation of the development of *in vitro* anti-retroviral resistance in HIV-1 isolates obtained from patients participating in the CPCRA Combination Nucleoside clinical trial.
5. The Johns Hopkins University/MACS studies.
6. The Johns Hopkins University seroconverter study.
7. The *in vitro* testing of experimental anti-retroviral compounds using HIV-1 isolates.
8. RV100 - This protocol is a phase 1 clinical trial to evaluate the safety and efficacy of autologous transfusion of CD4+ T-cells established as long term cultures by stimulation with antibodies to CD3 and CD28. This study resulted in the discovery of HIV inhibitory factors. The cellular phenotyping group performed a number of studies in support of this protocol including the isolation of virus, phenotypic characterization of the isolated viruses (SI/NSI determination) and a series of studies aimed at identifying the factors responsible for the HIV inhibitory effect and characterizing the phenomenon. These activities were discussed in some detail above.

The drug sensitivity assays performed on RV43 and RV65 isolates resulted in the determination of the *in vitro* drug inhibitory concentration of four anti-viral agents for each virus isolate tested. An example of a final report for an RV43 patient is presented in Table 19. During this period 268 drug sensitivity assays were performed and reported for RV43 patient isolates. Assays performed on the six patients enrolled in the RV65 study examined the *in vitro* resistance to AZT, ddC, ddI, and compound A. Thirteen assays were performed before this study was terminated.

For the additional studies, 187 CPCRA specimens were received and processed for virus isolation. It is anticipated that during the next fiscal year virus titration and drug sensitivity assays will be performed on these isolates. For the Johns Hopkins University MACS study the drug testing group received 52 vials of frozen cells from individual patients for virus isolation. Virus was isolated from 30 of these specimens and we were requested to determine the virus titration and *in vitro* resistance to AZT for 16 of these isolates.

For the Johns Hopkins University seroconverter study, we received 16 isolates for

Table-16: VIRUS ISOLATE NUMBER 102743

Date Received: 3/4/94

Date Virus Titration Set-up: 5/8/94

Titration Data

Assay	O.D.	Number of + wells per							Virus	4-Drug Plate Virus Dilution
Date	Cutoff	16	64	256	1024	4096	16384	65536	TCID ₅₀	Required
5/19/94	0.504	6	6	6	6	2	2	1	6472	0.530

Date Drug Sensitivity Set-up: 6/1/94

Date Drug Sensitivity Assayed: 6/8/94

Drug Sensitivity Data

AZT (uM)	p24x10 ⁵	Fraction Affected	ddC (uM)	p24x10 ⁵	Fraction Affected
0	3.69		0	3.69	
0.001	3.35		0.01	2.83	0.23
0.01	3.44		0.1	0.48	0.87
0.1	3.52	0.05	1.0	0.04	0.99
1.0	2.01	0.46			
5.0	0.25	0.93			

IC₅₀ = 0.8979

IC₅₀ = 0.0247

ddI (uM)	Fraction p24x10 ⁵ Affected		Compound A	Fraction p24x10 ⁵ Affected	
0	3.69		0	3.69	
0.1	3.33	0.10	0.03	3.63	0.02
1.0	2.86	0.22	0.3	0.09	0.98
5.0	2.46	0.33	1.0	0	
10.0	1.40	0.62	3.0	0	
25.0	0.07	0.98			

IC₅₀ = 2.5091

IC₅₀ = 0.0949

testing *in vitro* AZT resistance. Because of low virus titration results obtained for two of these isolates, assays could only be performed on 14 of these specimens.

Several experimental compounds were tested to determine their ability to inhibit replication of HIV isolates. These drugs were obtained from the laboratories of the Department of Applied Biochemistry of the Walter Reed Army Institute of Research, the Laboratory of Medicinal Chemistry at the National Cancer Institute, and private pharmaceutical companies. These compounds were tested by using isolates from RV43 patients and AZT-resistant and sensitive control virus isolates. A summary listing of all assays performed in support of antiviral drug susceptibility testing can be found in the appendix.

Development of a rapid drug screening assay

The current assays used to identify drug resistant HIV isolates require virus isolation, expansion and titration of the isolate followed by phenotyping. In order to reduce the time and cost, a rapid drug phenotype screening assays was developed. After a positive virus culture is confirmed, the procedure is as follows:

1. Resuspend the cells in the p24 positive culture (A1 Tube) and divide into three tube cultures each containing 0.800 ml of the resuspended cells. Label the tubes with SRA number and as AZT 0, AZT 0.2, or AZT 2.0
2. Into each of the three tubes, place 0.450 fresh co-cult media containing 2×10^6 PHA-stimulated PBMCs.
3. Into the tube labelled AZT 0, place 1.25 ml fresh co-cult media. Into the tube labelled AZT 0.2, place 1.25 ml fresh co-cult media containing 0.4 μ M AZT. Into the tube labelled AZT 2.0, place 1.25 ml fresh co-cult media containing 4.0 μ M AZT. Final volume of all three tubes should be 2.5 ml.
4. Continue to maintain the cultures using standard procedures. On Day 4 replace the media in the culture tubes with either co-cult media, co-cult media with 0.2 μ M AZT, or co-cult media with 2.0 μ M AZT. On Day 7, refeed the cultures with 2×10^6 PHA-stimulated PBMCs in the appropriate media, i.e. no AZT, 0.2 μ M AZT, or 2.0 μ M AZT. Save an aliquot of media from each tube on Day 4 and Day 7 refeeds for p24 assay.

The p24 (pg/ml) results of this assay using 22 isolates and comparison values to the conventional assay is shown in Table 23.

Table 23

Isolate	0	AZT Concentration (uM)		IC50 (uM) by ACTG/DoD
		0.2	2.0	
1	1024	0	0	0.1010
2	1136	0	0	0.0920
3	867	321	0	1.3947
4	1467	416	0	4.4568
5	1102	0	0	0.0206
6	1445	56	0	0.6692
7	1876	827	47	1.4245
8	1203	528	0	1.5471
9	2046	1002	116	2.3359
10	1422	0	0	0.1371
11	1127	0	0	0.0379
12	1876	18	0	0.1767
13	1443	0	0	0.0603
14	1154	139	0	0.5008
15	1889	477	0	0.2527
16	1322	264	18	0.5843
17	964	567	43	0.3595
18	1221	1316	867	>5.0000
19	1802	1765	675	4.4568
20	1556	556	110	1.6692
21	1677	627	97	2.6602
22	1765	760	119	1.3726

4. Data Group Section

The computer capabilities of contract DAMD17-92-C-2504 took a major leap forward during the contract's operation with the installation of a area-wide network encompassing, first, the Taft Court, Shady Grove, and Key West facilities of SRA and finally, subsequent to consolidation, a fully networked facility at 4 Research Ct. This process involved several steps, including upgrading of the RLIMS database hardware, installation of linked local area networks (LANs), provision of 386-type or better computers for all personnel and provision of new software packages. In addition, Direct communications between the Henry Jackson foundation computers were established on an interim bases for the uploading of data from RLIMS and a ccMail connection was put in place to facilitate direct communications between WRAIR and SRA personnel.

Establishment of Information System Hardware

The RLIMS database upgrade involved replacement of an aging 386-based 8-user SCO Unix-based host with a Sun SPARCserver 670, capable of supporting upwards of 100 users and accommodating 5 years of data growth. The response time of queries on the system improved 1000% in many instances. The data support group was originally consolidated at the Key West facility and then at 4 Research court, improving internal efficiency. Programming productivity was increased enormously due to the introduction of new high-speed equipment.

The second step involved linking the three sites internally with LANs and connecting those LANs via T-1 digital phone lines and microwave radio. Standard Windows-based applications were installed for universal access, including WordPerfect, Excel, and cc:Mail. Now, with the exception of the Macintosh, a user can perform any task from any workstation at any site. New computers were acquired for key personnel to replace 286 PCs, and eight computers were acquired for pooled use by lab technicians.

Introduction of New Software

1. Oracle Database - The Oracle database software was upgraded from Oracle v6 to ORACLE7. This improved data integrity and security and improved programming by enabling much of the data validation and processing logic to be coded into the database rather than the applications. SRA's Relational Laboratory Information Management System (RLIMS) utilizes Oracle and now resides on a Sun SPARC server located at 4 Research CT. The RLIMS Database contains patient identifiers, specimen information, freezer inventory, and results associated with the contract. The benefit of the RLIMS database being on the network is the ability for end users to access information concerning specimens they are assigned via Forms or Reports that reside on the RLIMS system. Investigation began into the acquisition of new graphical Windows/Mac front-end software to replace the character-mode Unix interface, and graphical LAN Architecture-end-user query software to enable scientists and lab technicians to build their own reports from the database without programming.

2. Excel Spreadsheet - Excel is the standard spreadsheet software package used on SRA's Lab Network. This package permits Lab supervisors as well as the technicians to analyze raw data produced by experimentally and stored in RLIMS. Currently raw data is either typed in or downloaded directly into a spreadsheet template for calculation or statistical evaluation. Once the spreadsheet is complete report formats including graphical charts can be applied and distributed through out the group via file sharing or our E-mail package CCMail.

3. CC Mail - This Software package allows contract personnel to communicate within their group and with others users on the network. This e-mail capability permits distribution of experimental protocols and data to all technical staff and interested parties without resorting to scheduled meetings. CCMail also supplies a central point for technicians to

store messages and files associated with tasks assigned by the laboratory supervisors. The Data Group worked closely with various SRA contract personnel to create procedures and programs within RLIMS to handle experimental plate data for all experimental work. The Data Group wrote a number of reports, and formulated methods to e-mail RLIMS output to contract personnel in Excel format.

4. Database management and reporting of Genotype Information - HIV sequencing or genotyping has proven to be a valuable tool both for the researcher and for the clinician in helping with drug therapy. This sequencing covers both the reverse transcriptase and the protease region of HIV. While we initially performed manual sequencing we have successfully switched exclusively to automated sequencing using the ABI 377XL automated DNA sequencer as described above. The advantages of this system is higher throughput and more reliable runs. An additional benefit is the "electronic " nature of the data. ABI provides propriety software for the analysis and alignment of the sequence data. This software was used for some of the initial reports allowing for crude sequence comparisons. Part of the problems with the ABI software is that it is a generic package for all sequencing projects. In order to generate a report more tailored to HIV geneotyping and more interpretable to clinicians we joined with the Data Management Department of SRA to develop a semi-automated reporting system for sequence data. The three main objectives of this project were: 1) storage of sequence data in the SRA RLIMS database, 2) Translation of nucleic acid sequence to protein sequence with proper handling of ambiguity codes, 3) Concise report generation with notification of mutations associated with known HIV drug resistance.

The program was written in Oracle and runs with our RLIMS database. The first objective was solved by the importation of a simple text file of the crude DNA sequence from the ABI software into the RLIMS database. In the database, as with other studies, the patient pertinent information along with the sequence data are linked. The importing of this data into the database also allows for a higher level of safety and security. The program next decodes the DNA sequence into an amino acid sequence using the universal genetic code. The program is also instructed to adequately handle ambiguity codes used to signify the presence of mixed bases. For example in the codon "ATR" the "R" is the code for either "A" or "G". The program would correctly translate this codon into a mixture of Isoleucine and Methionine. This information is essential to the proper interpretation of a patient's response to antiviral drugs.

An example of this final report is presented in Table 24 below. This DNA Sequence Mutation Report list all of the pertinent patient information at the top of the report along with the complete amino acid sequence. The protease and RT regions are handled as two separate reports but the features are identical. The power of the program is that it performs a amino acid by amino acid comparison of the test sample versus a standard (in this case it is sequence of the HIV-MN laboratory strain). All mutations are listed by position and actual mutation. Mutation know to be associated, compiled from the literature, with drug resistance are starred "*" to distinguished them from normal viral polymorphism. In the

Table 24
DNA Sequence Mutation Report

Study # A117 SRA # 53917 Type of Sequence SEQ_RT1 Obtained Date 16 Sep 97

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
P	I	S	P	I	E	T	V	P	V	K	L	K	P	G	M	D	G	P	r
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
V	K	Q	W	P	L	T	E	E	K	I	K	A	L	v	E	I	C	T	E
41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
M	E	K	E	G	K	I	S	K	I	G	P	E	N	P	Y	N	T	P	V
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
F	A	I	K	K	K	D	S	T	K	W	R	K	L	V	D	F	R	E	L
81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
N	K	r	T	Q	D	F	W	E	V	Q	L	G	I	P	H	P	A	G	L
101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
K	K	K	K	S	V	T	V	L	D	V	G	D	A	Y	F	S	v	P	L
121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
D	P	D	f	R	K	Y	T	A	F	T	I	P	s	I	N	N	E	T	P
141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160
G	I	R	Y	Q	Y	N	V	L	p	Q	G	W	K	G	S	P	ap	I	f
161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
Q	S	S	M	T	K	I	L	E	P	F	R	K	Q	N	P	D	m	v	I
181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200
c	Q	Y	M	D	D	l	Y	V	G	S	D	L	E	I	G	Q	H	R	ta
201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220
K	I	E	E	L	R	q	H	L	L	R	W	G	F	T	T	P	D	k	K
221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
H	Q	K	E	P	P	F	L	W	M	G	Y	E	L	H	P	D	K	W	T
241	242	243	244	245	246	247	248	249	250										
V	Q	P	I	V	L	P	E	K	D										

<< 9 Mutation(s) found >>

Amino Acid		
Original	Position	Mutation
K	20	R
I	35	V
K	83	R
K	122	P
A	158	A/P
I	178	M
Y	181	C *
A	200	T/A
R	207	Q

* Mutation potentially associated with drug resistance. See drug resistance table.

example report the RT shows a Y181C mutation , it is known that this is associated with resistance to the NNRTI Delapravdine. Likewise the protease shows a particular L90M mutation for resistance to sequinavir. This reporting mechanism has provided enhanced secure data storage as well as a concise and relevant report. Finally, the programs are written in a flexible format allowing tailor made reports and changes in specific parameters.

It should be noted that all software packages were upgraded in FY95-96 to include WordPerfect 6.0/6.1 for PC and 3.5 for Mac, Excel 5.0 for both platforms and the latest ccMail which now accomodates internet communications. Full internet access was also established in FY95 with the introduction of Netscape software and the construction of SRA's own home page on the world wide web. The homepage is still in the process of revision and should be completely functional in the early part of FY96.

At the request of WRAIR personnel and the contract office's representative, a procedure was established to pass all contract generated data to WRAIR via the Henry M. Jackson Foundation's (HMJF) computer network. The first data transfer encompassed the period January 1988 through July 1993. HMJF loaded the data into their Informix database for further processing at the discretion of WRAIR investigators. The data was transferred on 1/4" tape. Meetings were held to formulate plans for direct linking of the new SRA network with the WRAIR/HMJF computer network, allowing HMJF to access approved data downloads directly. HMJF agreed to procure and install the necessary equipment. Since SRA's consolidation, some changes in transfer procedures were required and the T1 line was terminated. Finally, new programming capability has been added and staffing increased to accomodate the workload.

All told, during the period encompassed by this contract (10/1/92-9/30/95) SRA has spent close to one million dollars on an upgraded computer facility which has significantly improved communications and data flow between WRAIR and SRA principal investigators.

SUMMARY

A number of significant contributions to WRAIR's mission were made by this contract during the three base years from Oct. 1992 thru the end of Sept. 1995 and during the final two option years despite the significant reduction in funding and the resulting loss of staff. Included is the optimization, validation and extension of the 215 ARMS assay for genotypic resistance patients in a large-scale, nationwide clinical trial to assess the significance of this mutation. Comparison of the genotypic approach with the classical phenotypic assays of drug resistance described in the previous sections will undoubtedly demonstrate the usefulness of the genotypic assay for clinical management of patient therapy. During the contract period SRA also developed an ARMS assay for codon 74 mutations associated with DDI resistance and codon 184 associated with 3TC resistance. In addition to the qualitative 215 assay currently available, SRA worked independently to establish a procedure for the quantitative determination of RT mutations so as to discriminate mixtures and thus enhance the physcians ability to identify patient requirements for treatment

modifications. This assay was completed in FY96 with funding contributed by SRA and is available to the DoD on request.

In addition to the mutational assay, SRA put in place manual sequencing procedures for HIV reverse transcriptase, developed primers and procedures for env sequencing and, thru WRAIR's collaboration with the AIDS Clinical Trials Groups, began efforts to bring protease sequencing online. During the last base fiscal year, a time when funding was sharply reduced and the program was itself in jeopardy, SRA established an automated sequencing facility to enhance the capabilities already available to the government. These capabilities will permit WRAIR to evaluate resistance mutations and determine their significance on a patient-by-patient basis regardless of the test drugs employed. Automated sequencing protocols were refined during the option years and a number of diagnostic sequencing requests were processed as a prelude to the start of the GART (genotyping for antiretroviral therapy) protocol which is a DoD-CPCRA collaboration to evaluate diagnostic sequencing for patient management. These pre-GART studies were initiated to standardize and validate the sequencing protocol to be employed in the GART study.

In the molecular area, the analysis of viral burden has become the principle surrogate marker of drug efficacy. A number of assays, commercial and in-house have been offered for quantitation of this marker including Roche's Amplicor, NASBA, bDNA, etc. At the request of the COR, SRA became certified to run the Roche assay and trained two staff members who were validated by Roche and the ACTG to provide this service. SRA now provides RT-PCR quantitation for diagnostic work performed by WRAIR for its patients. In FY96 SRA trained additional personnel to ensure continued support for this area of research.

SRA, thru the cell phenotype working group provided services for the isolation, expansion and titration of isolates employed for serotyping and neutralization. Although demand for this service has declined, because of cost considerations, it remains available to all WRAIR investigators. As a result of the problems in funding the cellular phenotype working group initially shifted emphasis to studies of gene therapy. We developed an *in vitro* assay for the evaluation of antiviral gene constructs in established cell lines and have been actively pursuing a system that will permit efficacy analysis in PBMCs with primary isolates. Such a system will prove invaluable for the longterm culture and ex-vivo treatment of patient cells with antiviral genes. Of particular note is the rather surprising discovery, made at SRA in collaboration with investigators at the Navy's Medical Research Institute in Bethesda, that CD3/CD28 antibody solid phase (in cis) stimulation yields a cell population reversibly resistant to HIV infection or transmission and that this cell population actively transmits this suppression, by way of a soluble factor(s), to cells normally susceptible to infection. This work suggests the existence of a heretofore unknown mechanism of resistance to HIV infection and points toward new methodologies for the treatment of infected individuals. This represents a major contribution by WRAIR and SRA to the immunobiology and therapy of HIV. Work continued on this project thru the two option years and yielded important information concerning the range of antiviral activities found in the supernatants of CD3/CD28 antibody stimulated cells. Of major importance is the demonstration that these factor(s) can act in combination with clinically approved antiviral

drugs to enhance protection. This work continues in the absence of DoD support, but in collaboration with WRAIR (NMRI, HMJF) personnel with funding provided by SRA Technologies.

During the three base years of this contract the drug testing group supported a large number of clinical protocols including RV43, RV65, RV77 and RV79 in addition to the ACTG's CPCRA studies. Again because of funding restrictions in the two option years, the drug testing group sharply reduced efforts to test newly developed putative antiviral agents. However, support for a number of protocols, requiring drug susceptibility phenotyping continued and SRA participated with this contract's COR in the ACTG's drug resistance SWAT teams and have been instrumental in the development of an improved system for the rapid and cost effective phenotypic analysis of resistance to antiviral drugs as described earlier in this report.

Finally, SRA has made great strides in its continuing effort to provide computer support and data communications to WRAIR and its collaborators. More than one million dollars has been spent to improve SRA's computing infrastructure and upgrade its programing staff. New software has been put in place to support WRAIR's genotyping efforts and to distribution of reports.

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APPENDIX

I Publications and Abstracts for FY95, FY94 and FY93

Papers (SRA Staff participants in bold face type)

1. Carroll RG., Riley JL., Levine BL., Feng Y., Kaushal S., Ritchey DW., Bernstein W., **Weislow OS.**, Brown CR., Berger EA., June CH., St. Louis DC. Differential regulation of HIV-1 fusion cofactor expression by CD28 costimulation of CD4+ T cells. Science 1997 Apr 11;276(5310):273-6
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Abstracts

1. **Weislow, S. Snyder, S. Belay**, B.L. Levine, J. Riley, R. Carroll, D. St Louis and C. June, Factors Produced by CD3/CD28 Antibody-Stimulated CD4+ T-Cells Inhibit Replication of SI Isolates of HIV and Potentiate The Activity of Antiviral Drugs. Presented at the 4th annual meeting of the Institute of Human Virology, September, 1997, Baltimore MD.
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6. Mayers DL, Yerly S, Perrin L, Imrie A, Cooper DA, Karney WW, Brown AE, Rakik A, Harris R, Gambel J, **Weislow OS**, Lennox JL, Burke DS: Prevalence and clinical impact of seroconversion with AZT-resistant HIV-1 between 1988 and 1994. 2nd National Conference on Human Retroviruses and Related Infections, Washington, DC, 1995.
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10. Mayers DL, Wagner KF, Chung RCY, Vahey MT, **Weislow OS**, Zhou S, Burke DS, RV43 Study Group: Virologic Response to ddI following prolonged AZT Rx. Xth International Conference on AIDS, Japan, Abstract 359B, 1994.
11. Mayers D, Wagner KF, Chung RCY, Vahey MT, **Weislow OS**, Lennox JL, Zhou SYJ, Burke DS: Relationship of SI/NSI status to development of ZDV resistance and clinical outcomes in patients on ZDV therapy. HIV Drug-Resistance Third International Workshop, Kauai, Hawaii, 1994.
12. Mascola JR, **Weislow O**, **Snyder S**, et al. Comparison of Genetic Subtype to Neutralization Serotype for Human Immunodeficiency Virus Type-1. (1994 Keystone Symposia: Prevention and Treatment of AIDS.) J Cell Biochemistry 1994;18B [Abstract J321].
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16. Chiang, P.T., Joshi, B., Hewlett, I.K., **Lane, J.R.**, Doctor, B.P., Burke, D.S. and Mayers, D.L., 3-Deaza Adenosine Analogs as Novel Anti-HIV Drugs. for presentation at the Amer. Soc. of Pharmacol. & Exptl. Therapeutics Meeting in San Francisco, CA July 30, 1993.
17. Mascola, J., **O. Weislow, S. Snyder, S. Belay**, F. McCutchan, J. McNeil, D. Burke and M.C. Walker. Neutralizing Antibody Activity in Sera from Human Immunodeficiency Virus Type-1 Vaccine Recipients from the AIDS Vaccine Clinical Trials Network. Sixth NCVDG Meeting, Conference on Advances in AIDS Vaccine Development, Washington D.C., 1993.
18. Mayers, D.L., Wagner, K.F., Chung, R.C.Y., **Lane, J.R.**, Vahey, M.T., **White, F.A.**, Ruiz, N.M., Hicks, C.B., **Weislow, O.S.**, Gardner, L.I., Burke, D.S. and the RV43 Study Group, Zidovudine (AZT) Resistance is Temporally Associated with Clinical Failure in Patients on AZT Therapy. for presentation at the First National Conf. on Human Retroviruses and Related Infections, Washington, D.C. Dec. 1993.
19. Mayers DL, Wagner KF, Chung RCY, Lane JR, Vahey MT, **White FA**, Ruiz NM, Hicks CBN, **Weislow OS**, Gardner LI, and Burke DS. Zidovudine (AZT) resistance is temporally associated with clinical failure in patients on AZT therapy. First National Conference on Human Retroviruses and Related Infections, December 1993, Washington D.C.
20. Mayers D, Erice A, **Lane J**, McCutchan F, Jabs D, Tudor-Williams G, Sannerud K, **Weislow O**, Page S, Felder K, and Balfour H. Foscarnet (FOS) resistant HIV isolates emerge during extended FOS therapy for CMV retinitis. 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, 1993.
21. Mosca JD, Kaushal S, Larussa V, Kessler S, Gartner S, Kim J, Perera P, Yu Z, Ritchey D, Xu J, Rosenberg Y, St Louis D, **Weislow O**, Mayers D and Burke D. Human bone marrow-derived CD34+ cells as targets for gene therapy against HIV infection. Second National Conference on Human Retroviruses and Related Infections, December 1994, Washington D.C.
22. **White, F.A.**, Tran, M., Zhao, X., Antezana, M., **Weislow, O.**, Vahey, M. and Mayers, D.L., An Improved 215 PCR Assay for DNA and RNA. presented at the Third Workshop on Viral Resistance Gaithersburg Hilton, Sept 19-23, 1993.
23. **Weislow, O.S.**, **S. Belay, S. Snyder, M. Yeager**, F. McCutchan, J. Mascola and D.L. Mayers. Optimization of Peripheral Blood Mononuclear Cell (PBMC)-Based Neutralization Assays for Early Passage Clinical Isolates of HIV-1. Sixth NCVDG Meeting, Conference on Advances in AIDS Vaccine Development, Washington D.C., 1993.

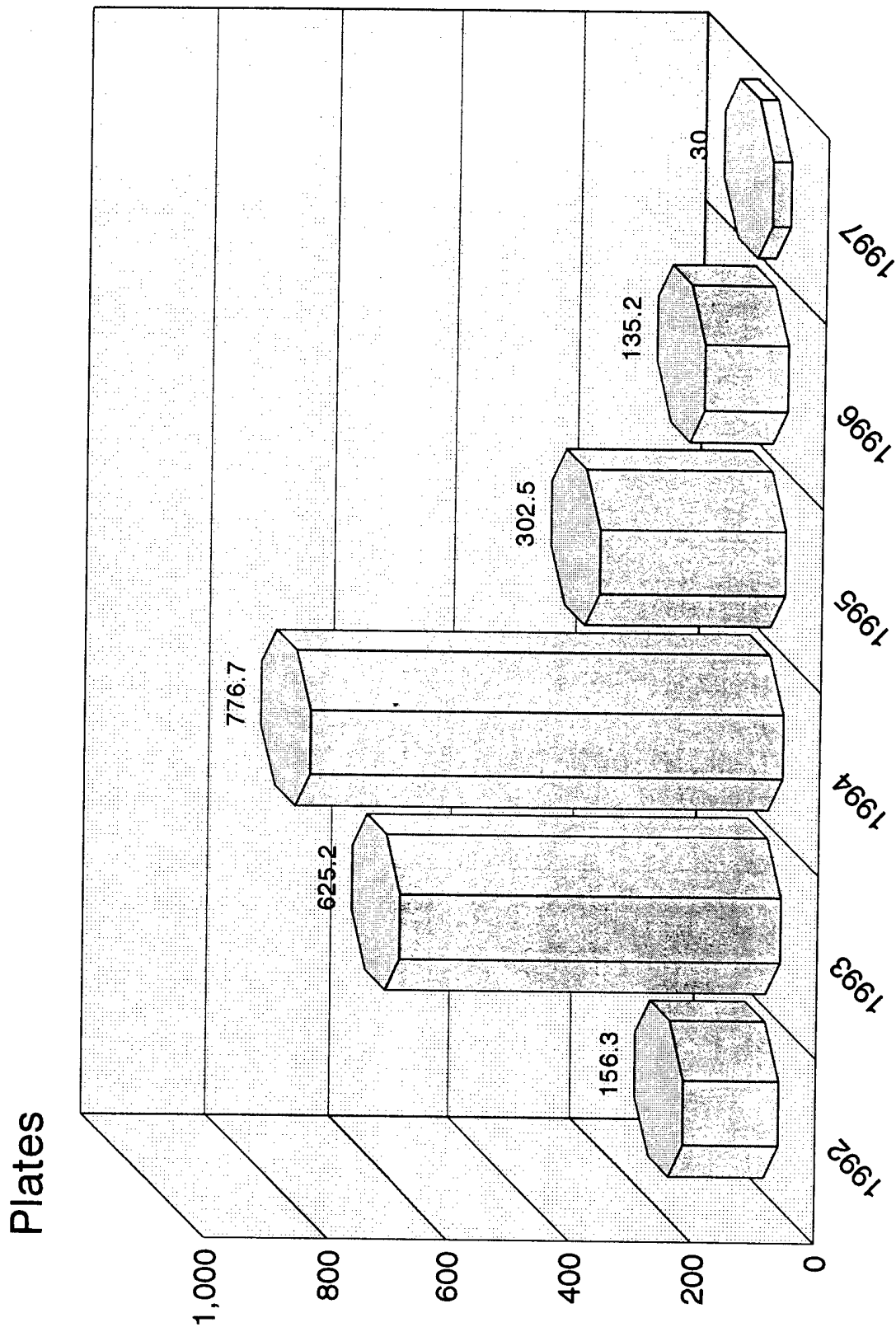
II Summary of Services Provided

SERVICES PERFORMED		NUMBERS PERFORMED					
		FY93	FY94	FY95	FY96/OpYr	FY97/OpYr	TOTAL
RV43	Specimens Received	919	420	10	40	0	1389
	Cocultures setup	491	263	10	129	0	893
	Successful Virus Expansions	253	128	18	0	0	399
	Virus Titrations	10416	5376	0	0	0	15792
	4-Drug Assays	11286	5130	0	0	0	16416
	Syncytium Induction Assays	723	1668	54	0	0	2445
	RNA and DNA 215-Point Mutation Assays	0	1083	0	0	0	1083
RV79	Specimens Received	0	367	1168	1094	207	2836
	Cocultures setup	0	52	292	69	0	413
	Successful Virus Expansions	0	24	132	0	0	156
	Virus Titrations	0	1386	4830	0	0	6216
	4-Drug Assays	0	1881	6555	0	2	8438
	Syncytium Induction Assays	0	87	327	0	0	414
	Viral Burden analysis for substudy	0	0	0	250	0	250
	Sequencing for substudy	0	0	0	0	49	49
	RNA and DNA 215-Point Mutation Assays	0	188	600	0	0	788
CPCRA	Specimens Received	314	493	490	37	0	1334
	Cocultures setup	283	463	488	0	0	1234
	Successful Virus Expansions	106	193	160	0	0	459
	Virus Titrations	0	294	1176	0	0	1470
	4-Drug Assays	0	0	1425	0	0	1425
	RNA and DNA 215-Point Mutation Assays	0	0	0	0	0	0
	Viral Burden: Roche Amplicor	0	0	250	0	0	250
	Pre GART Sequencing Standardizations	0	0	0	50	100	150
Int'l. Sero.	Specimens Received	19	22	9	5	0	55
	Cocultures setup	32	41	14	0	0	97
	Successful Virus Expansions	29	26	13	0	0	68
	Virus Titrations	1344	1722	588	0	0	3654
	4-Drug Assays	0	0	1254	16	0	1270
	Virus Infectivity Reduction Assay	0	0	12	0	0	12
Virus Immunotyping and Immunoneutralization	Virus Titrations--AVEG	0	294	0	0	0	294
	Neutralization Screening--AVEG	0	84	0	0	0	84
	Serum Titration--AVEG	0	870	0	0	0	870
	Virus Expansions--AVEG	0	7	0	0	0	7
	Virus Titrations--WHO	0	336	0	0	0	336
	Neutralization Screening--WHO	0	96	0	0	0	96
	Serum Titration--WHO	0	420	0	0	0	420
	Virus Expansions--WHO	0	20	0	0	0	20
	Virus Titrations--R&D	0	4116	0	0	0	4116
	Neutralization Screening--R&D	0	30	0	0	0	30
	Serum Titration--R&D	12	360	0	0	0	372
	Virus Expansions--R&D	0	98	0	0	0	98
	Syncytium Induction Assays	0	42	0	0	0	42
Antiviral Gene Therapy Evaluation	Cell Culture Maintaine	0	104	24	0	0	128
	Mycoplasma Evaluation and Treatment	0	57	12	0	0	69
	Virus Expansions	12	16	2	0	0	30
	Antiviral Gene Evaluations	0	104	38	0	0	142
	Reverse Transcriptase Assays	0	2	2	0	0	4

SERVICES PERFORMED		NUMBERS PERFORMED					
		FY93	FY94	FY95	FY96/OpYr	FY97/OpYr	TOTAL
Other	Virus Expansions	0	0	23	30	40	93
	4-Drug Assays for Dr. Chaing	0	0	8	18	8	34
	4-Drug Assays for Lynx	0	0	22	0	0	22
	4-Drug Assays for Dr. Mellors	0	0	2	0	0	2
	4-Drug Assays for Dr. Schmidt	0	0	28	0	0	28
	215 for seroconverters	0	143	0	0	0	143
	RV77, Dr. Robb, Facs, etc	0	164	0	0	0	164
	DNA Sequencing (misc)	0	28	13	50	60	151
	Molecular Biology R&D Assays Required	0	650	0	75	100	825
	RV100 + R&D Support for Dr. June						
	Specimens Received	0	0	0	8	119	127
	Virus isolations	0	0	0	2	38	40
	codon 215 ARMS assays	0	0	0	0	76	76
	SI/NSI Determinations	0	0	0	0	2	2
	Cultures established for inhibitor tests	0	0	0	0	2500	2500
	Virus expansions for NMRI stocks	0	0	0	0	3	3
	Virus Stocks provided (vials)		25	50	50	200	325
	ARMS Assays for 215 & 184	0	0	0	150	160	310
	Support Services						
	p24/Tissue cultures (misc)	65646	65243	25299	6629	1170	163987
	Repository: Vials added	14674	15657	14509	6884	4680	56404

AC Plate Totals

490 CONTRACT OCT92 - AUG97



III Personnel Participating On The Contract

Job Number	Job Item	Employee Number	Employee Name
J 490	49000	114	SONYA DILWORTH
J 490	49000	116	
J 490	49000	132	MAURICE L. EDWARDS
J 490	49000	132	
J 490	49000	186	CHRISTOPHER H. HAMILTON
J 490	49000	186	
J 490	49000	269	PAUL W. KRIEBEL
J 490	49000	269	
J 490	49000	568	TERRY B. SHARP
J 490	49000	568	
J 490	49000	569	LINDA I. MERRITT
J 490	49000	569	
J 490	49000	580	TERMINATED CONV. John Wang
J 490	49000	580	
J 490	49000	607	TERMINATED CONV. Lisa Delenga
J 490	49000	607	
J 490	49000	614	BRIAN M. MC DIVERN
J 490	49000	614	
J 490	49000	623	TERMINATED CONV. Ji Seok Yoon
J 490	49000	623	
J 490	49000	639	DAVID W. BRANTLEY
J 490	49000	639	
J 490	49000	648	TERMINATED CONV. Felicia D. Carpenter
J 490	49000	648	
J 490	49000	657	CHRISTINE A KOCH
J 490	49000	657	
J 490	49000	676	TOMMY R. SPARKS, JR.
J 490	49000	676	
J 490	49000	697	DECHENG CAI
J 490	49000	697	
J 490	49000	700	TERMINATED CONV. Orion Robinson
J 490	49000	700	
J 490	49000	701	MY NGOC TRAN DO
J 490	49000	701	
J 490	49000	706	TERMINATED CONV. Ewa Turner
J 490	49000	706	
J 490	49000	714	TERMINATED CONV. James M. Guntow
J 490	49000	714	
J 490	49000	717	KILIN ZHAO
J 490	49000	717	
J 490	49000	720	KEVIN M. GOLD
J 490	49000	720	
J 490	49000	724	TERMINATED CONV. Kelly J. McClellan
J 490	49000	724	
J 490	49000	726	TERMINATED CONV. Marcia Webb
J 490	49000	726	
J 490	49000	729	NANCY E. MARSHALL
J 490	49000	729	
J 490	49000	733	SEIFU M. RELAY
J 490	49000	733	

Job Number	Job Item	Employee Number	Employee Name
J 490	49000	733	JEFFREY A. SITZLAR
J 490	49000	744	
J 490	49000	744	JANICE M. DARNEN
J 490	49000	745	
J 490	49000	745	JAMES R. LANE
J 490	49000	750	
J 490	49000	756	REBECCA ERELING
J 490	49000	756	
J 490	49000	759	GREGORY A. JASPER
J 490	49000	759	
J 490	49000	763	BUSAN R. NELSON
J 490	49000	763	
J 490	49000	774	TERMINATED CONV. Lara Skutyskie
J 490	49000	774	
J 490	49000	783	TERMINATED CONV. Hla Kyaw
J 490	49000	783	
J 490	49000	790	GIN ZHOU
J 490	49000	790	
J 490	49000	791	CARLA R. CECCARELLI-GLASSER
J 490	49000	791	
J 490	49000	799	TERMINATED CONV. Frank White III
J 490	49000	799	
J 490	49000	800	TERMINATED CONV. Cheryl Price
J 490	49000	800	
J 490	49000	809	TERMINATED CONV. David Parrish
J 490	49000	809	
J 490	49000	813	MARK VOELKNER
J 490	49000	813	
J 490	49000	819	DIANE L. PLETOVICH
J 490	49000	819	
J 490	49000	821	ROBERT GUNTOW
J 490	49000	821	
J 490	49000	825	TERMINATED CONV. Kendall Price
J 490	49000	825	
J 490	49000	826	SHARON A. BARGO
J 490	49000	826	
J 490	49000	832	JOHN M DYE JR
J 490	49000	832	
J 490	49000	837	CRAIG J. DESARIO
J 490	49000	837	
J 490	49000	844	ROBERT C. EELKEMA
J 490	49000	844	
J 490	49000	853	CHRISTINE LYNN BASSO
J 490	49000	853	CHRISTINE LYNN BASSO HUNT
J 490	49000	853	
J 490	49000	857	TERMINATED CONV. Mary E. Kanne
J 490	49000	857	
J 490	49000	858	OWEN S. WEIBLOW
J 490	49000	858	

10/14/97 11:38:11 J 490 DIRECT LABOR 09/30/92 - 09/26/97 PAGE 3

Job Number	Job Item	Employee Number	Employee Name
J 490	49000	858	
J 490	49000	861	TERMINATED CONV.
J 490	49000	861	
J 490	49000	868	
J 490	49000	868	LISA STRAIT-HUGHES
J 490	49000	868	TERMINATED CONV.
J 490	49000	870	
J 490	49000	870	TERMINATED CONV.
J 490	49000	871	
J 490	49000	871	DIANMEN PAN
J 490	49000	879	
J 490	49000	879	ASHLEY HUNT
J 490	49000	879	
J 490	49000	884	TERMINATED CONV.
J 490	49000	884	
J 490	49000	886	BARRY PHELPS
J 490	49000	889	
J 490	49000	889	TERMINATED CONV.
J 490	49000	889	
J 490	49000	890	STEWART W. SNYDER
J 490	49000	890	
J 490	49000	893	JOSEPH A. RENFRO
J 490	49000	893	
J 490	49000	895	TERMINATED CONV.
J 490	49000	895	
J 490	49000	896	MIRIAM V. CRUZ
J 490	49000	896	
J 490	49000	899	ADRIAN K. MORIS
J 490	49000	899	
J 490	49000	901	JOHNETTA M. DAVIS
J 490	49000	901	
J 490	49000	905	TERMINATED CONV.
J 490	49000	905	
J 490	49000	906	TERMINATED CONV.
J 490	49000	906	
J 490	49000	908	TERMINATED CONV.
J 490	49000	908	
J 490	49000	909	ANTHONY S. CLARK
J 490	49000	909	
J 490	49000	911	TERMINATED CONV.
J 490	49000	911	
J 490	49000	916	TERMINATED CONV.
J 490	49000	916	
J 490	49000	921	NIMBERLEY LIZIK
J 490	49000	921	
J 490	49000	924	KALACHAR BURYANARAYANA
J 490	49000	924	
J 490	49000	925	KATHY M. BANUEL
J 490	49000	925	
J 490	49000	927	PAULA M. TEUFEL
J 490	49000	927	

Job Number	Job Item	Employee Number	Employee Name
J 490	49000	927	
J 490	49000	929	ANNMARIE SCHRAMM
J 490	49000	929	
J 490	49000	930	LEWIS B. DAVIS
J 490	49000	930	
J 490	49000	933	TERMINATED CONV.
J 490	49000	933	
J 490	49000	934	STEPHANIE L. ROBE
J 490	49000	934	
J 490	49000	938	RANDOLPH D. CALDWELL
J 490	49000	938	
J 490	49000	939	MICHAEL S. RENDLER
J 490	49000	939	
J 490	49000	948	STAFFORD V. KEELS
J 490	49000	948	
J 490	49000	949	JAROSLAW KALKA
J 490	49000	949	
J 490	49000	950	KRISTIN D. WILSON
J 490	49000	950	
J 490	49000	951	BRENDA K. MILLER
J 490	49000	951	
J 490	49000	964	PAULINE J. BROWN
J 490	49000	964	
J 490	49000	970	LISA M. GERBER
J 490	49000	970	
J 490	49000	978	GUERRA R. BENBERRY
J 490	49000	978	
J 490	49000	981	JOHN T. KOBIK
J 490	49000	981	
J 490	49000	983	DARRELL L. COLLIER
J 490	49000	983	
J 490	49000	985	MATTHEW R. EAGEN
J 490	49000	985	
J 490	49000	986	DAVID C. FRITZINGER
J 490	49000	986	
J 490	49000	988	CRYSTAL A. JEFFREYS
J 490	49000	988	
J 490	49000	1,000	MARGARET A. MAURICE
J 490	49000	1,000	
J 490	49000	1,001	KIMBERLY M. VALENTINO
J 490	49000	1,001	
J 490	49000	1,009	BRENDA B. SYKES
J 490	49000	1,009	
J 490	49000	1,012	RICHARD A. TUBING
J 490	49000	1,012	
J 490	49000	1,022	SUSAN W. JONES
J 490	49000	1,022	
J 490	49000	1,023	CHRISTOPHER T. WRIGHT
J 490	49000	1,023	

Job Number	Job Item	Employee Number	Employee Name
J 490	49000	1,030	LAUREN K. HUTTON
J 490	49000	1,030	
J 490	49000	1,034	J. ERIC HAUX
J 490	49000	1,034	
J 490	49000	1,035	TASHA M. BLANKS
J 490	49000	1,035	
J 490	49000	1,036	AUDREY R. CHAMBERS
J 490	49000	1,036	
J 490	49000	1,037	BETH P. DILLEHAY
J 490	49000	1,037	
J 490	49000	1,038	SHARON L. HENNE
J 490	49000	1,038	
J 490	49000	1,050	SAMUEL C. CHATMAN JR
J 490	49000	1,050	SAMUEL C. CHATMON JR
J 490	49000	1,050	
J 490	49000	1,051	LAUREN C. HALLEY
J 490	49000	1,051	
J 490	49000	1,052	DANIEL L. BALLAHAN
J 490	49000	1,052	
J 490	49000	1,055	JACQUELINE CHILDS
J 490	49000	1,055	
J 490	49000	1,064	FOON K. LEE
J 490	49000	1,064	
J 490	49000		

*** END OF REPORT ***