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MECHANISM OF CYTOTOXICITY OF THE AIDS VIRUS, HTLV-III/LAV

ANNUAL REPORT

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The study was undertaken in order to devise a quantitative assay of human immunodeficiency virus type 1 (HIV-1) load in patients' tissues. No methods are currently available which are both sensitive and specific for this purpose. Our method utilizes blood mononuclear cell or tissue DNA. HIV-1 sequences are amplified by the primer chain amplification reaction (PCR) technique. The reaction products are detected and quantitated by polyacrylamide gel electrophoresis, ethidium bromide stain, and Southern blot hybridization. This method has proven succesful using a single set of primers in detection of HIV-1 DNA sequences from 10 of 12 HIV-1 infected individuals, and 0 of 10 uninfected individuals. Methods of quantitation of using standard curves with defined amounts of HIV-1 DNA sequences and internal controls are under investigation. In addition, we have developed a sensitive and specific assay from HTLV-1 and HTLV-II DNA sequences using the same methodology and we have successfully applied it to the evaluation of blood samples from asymptomatic HTLV-1 infected individuals and individuals with adult T cell leukemia-lymphoma.

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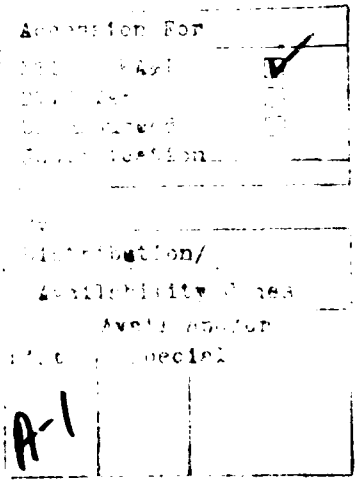
Foreword

1) Citations of commercial organizations and trade names in this report do not constitute an offical Department of the Army endorsement or approval of the Army endorsement or approval of the products or services of these organizations.

2) For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46.

3) The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Moleclues (April 1982) and the Administrative Practices Supplements.



Background and Significance

HIV-1 infection may cause no symptoms, an acute influenza-like illness, lymphadenopathy syndrome, acquired immunodeficiency disease syndrome (AIDS)-related complex (ARC) (including night sweats, fever, diarrhea, weight loss, oral candidiasis), or AIDS (including neurological disease, opportunistic infections, or malignancies) (1). A significant number of infected individuals will progress from asymptomatic to symptomatic stages of disease (2). There is considerable data to suggest that virus load and immunosuppression is a major determinant of disease stage (3,4). Immunosuppression is likely directly related to virus load as suggested by both in vitro studies as well as clinical studies (4,5). Thus measurements of virus load are likely to be critical for prognostic determinations.

There are currently no sensitive and specific methods of estimating virus load. HIV-1 lymphoblastoid culture results are not closely related to the inoculum dose over several orders of magnitude (6). HIV-1 plasma culture technique are subject to the same criticisms; in addition, their lack of sensitivity further compromises their routine use. Soluble HIV-1 p24 antigen measurements using serum, alcoholprecipitated serum, or cerebrospinal fluid lack adequate sensitivity to detect viral products in more than 10-30% of all HIV-1 infected individuals (7). Direct analysis of HIV-1 DNA is blood mononuclear cells or tissues by Southern blot hybridization is capable of detecting viral sequences in only about 15% of cases (8); its limit of sensitivity of about 1 DNA sequence per 100 cells is inadequate for evaluation of HIV-1 infected materials. Direct analysis of cellular or free HIV-1 RNA sequences by hybridization methods also lacks adequate sensitivity for this purpose (9). In situ RNA hybridization is likely to be a highly sensitive and specific technique, but its technical difficulties make routine clinical use impossible (10).

The lack of adequate assays of virus load has seriously compromised the effectiveness of developing anti-viral therapies for HIV-1. HIV-1 cultures and p24 antigen measurements are used to monitor anti-viral efficacy in clinical trials (11). However, the lack of sensitivity and the biological variation in these measures make it difficult to rely on these data in assessing therapeutic efficacy. Thus, investigators must also rely on indirect immunological (e.g. T4 lymphocyte count, delayed hypersensitivity reaction) and clinical (e.g. development of opportunistic infections or neoplasms or death) criteria for monitoring such studies. A sensitive, specific, quantitative assay of HIV-1 virus load will signficantly increase the efficiency of evaluation of anti-viral agents, both in terms of time, cost, and patient

morbidity and mortality.

A novel technique was developed in Dec., 1985 by Saiki and coworkers for amplification of DNA sequences (12). This technique is known as primer chain amplification reaction (PCR). DNA extracted from any tissue may be utilized in this assay. The two strands of DNA are denatured with heat. Specific oligonucleotides identical and complementary to sequences of 20-30 nucleotides on the 5' and 3' ends, respectively, of a DNA sequence of 100-2000 nucleotides are then annealed to the denatured DNA. A new strand of DNA is synthesized with a DNA polymerase. Thus after a single cycle of denaturation-annealing-polyerization, two copies of the sequence of interest are present. After two cycles, four copies are present, and after n cycles there are

$$(1 + k)^n$$

copies, where k is the efficiency of amplification at each cycle and ranges from 0 to 1. With a k value of 0.6, there are predicted to be 100,000 copies after 25 cycles. With a k value of 0.8, there are predicted to be about one million copies after 25 cycles.

Given the data from Southern blot hybridization directly from fresh tissues of HIV-1 infected individuals, we can estimate that on average there is about 1 DNA sequence per 300 cells, and that over 95% of individuals will have at least 1 DNA sequence per 1000 cells. The PCR technique should therefore allow amplification of rare HIV-1 sequences from undetectable to detectable levels. We have confirmed this prediction, as has several workers at Cetus in work published within the last year (13). However, the basic goal of quantitation of HIV-1 DNA sequences remains to be demonstrated and will be described further below.

Experimental Procedures and Results

We have used a variety of oligonucleotide primer pairs for amplification, including those with the 5' long terminal repeat sequences (LTR), leader sequence, gag, or env genes. The positions of the oligonucleotides in regions other than env are shown in Fig. 1; oligonucleotides within env include SK68 and SK69 and have previously been described (13). These oligonucleotides were chosen by the following criteria:

a) Oligonucleotides of 17-24 nucleotides were chosen to insure annealing. The longer oligonucleotides were designed for experiments using the Thermus aquaticus (Taq I) polymerase in which case polymerization is carried out at 72 degrees C. Thus, stable annealing would be required at this temperature.

b) Oligonucleotides were designed to have a GC content of at least 50% to insure stable hybridization.

c) Oligonucleotides were chosen which flanked regions of 100-300 nucleotides. The efficiency of the amplification reaction declines significantly when amplifying larger regions of DNA.

d) Oligonucleotides were chosen which are identical or complementary to HIV-1 DNA sequences which are theoretically and/or empirically conserved among different HIV-1 isolates. This will increase the liklihood that a given set of primers will anneal to HIV-1 DNA sequences from a variety of different isolates. These regions include those surrounding the TATA box, the polyadenylation signal, the tRNA primer binding site, and the beginning of the gag gene which encodes a myristylated p17 protein.

e) Oligonucleotides were not made which are identical or complementary to regions of the HIV-1 genome which are likely to have homology with cellular sequences. Thus, sequences within pol which have homology to possible endogenous human retroviruses were not utilized.

In the initial work PCR was performed with E. coli DNA polymerase I Klenow fragment and the amplifications cycles were performed by hand. Subsequent improvements in the technique which were incorporated later included the following:

a) Taq I polymerase became available in 11/87 and was substituted for Klenow polymerase at that time.

b) An automated PCR machine became available in 3/88 and was substituted for manual reactions at that time.

These improvements increased the speed of assays by a factor of about 50-fold and increased the efficiency of amplification.

In the initial experiments with Klenow polymerase, we demonstrated that with 20 cycles of amplification, using primers R13 and R14, cloned HIV-1 sequences could be amplified 10,000-fold. Thus, the efficiency per cycle is about 60%. Digestion of the cloned DNA with Sac I, which cuts between the two primer binding sites, reduced the efficiency of amplification dramatically. The size of the amplified product was 188 nucleotides, as predicted based on the sequences from this clone.

Using the same primer, amplification could be achieved with 1 microgram of HIV-1 infected H9 cell line DNA. In this case, amplification of more than 1000-fold was acheived as demonstrated by serial dilutions of the amplified product and slot blot hybridization analysis. The amplified product was 188 nucleotides in this case, as well. No HIV-1 DNA could be detected after amplification reactions using uninfected H9

cell DNA or MT2 DNA. The latter cell line is infected with HTLV-I but not HIV-1. HIV-1 DNA sequences could be detected after amplification using DNA derived from a peripheral blood mononuclear cell culture infected with a distinct isolate, JF.

In work with primers P2 and P3 and the Taq I polymerase, using 25 PCR cycles (denaturing at 94 degrees C for 2.5 min., annealing at 40 degrees C for 3 min., and polymerizing at 72 degees C for 2 min.) an amplified product of 223 nucleotides was obtained using 1 ng of cloned DNA. If a third primer, P4, was added, two different regions could be amplified simultaneously, yielding products of 129 and 223 nucleotides as predicted by available sequence data. However, if one adds a primer, R12, which is complementary to P3, amplification reactions are blocked, thus demonstrating the specificity of the technique.

Similar results were obtained with the Taq polymerase with cell line DNA as previously described in experiments utilizing the Klenow polymerase. Using primers P2 and P3, an amplified product of 223 nucleotides could be obtained from DNA of HIV-1 infected H9 cells, but not DNA from uninfected H9 cells.

The assay was then applied to DNA from fresh tissues of patients, in which the HIV-1 DNA sequence concentration is expected to be significantly lower. Tissues sources included samples from peripheral blood mononuclear cells, brain, lymph node, or spleen. Using primers P2 and P3, and the Taq polymerase, an amplified product of 223 nucleotides was obtained in tissues from 10 of 12 HIV-1 infected patients. HIV-1 DNA sequences could be detected in the amplified products of samples from all types of tissues described above, including samples that were negative by direct Southern blot hybridization analysis. The PCR assay performed with a single set of primers failed to amplify HIV-1 DNA sequences from two different brain samples of one HIV-1 infected patient or from lymph node and spleen samples from a second HIV-1 infected patient. No amplified HIV-1 DNA sequences were detected in any of the nine HIV-1 negative patients evaluated, demonstrating the specificity of the reaction.

Under the reaction conditions described above, a similar quantity of HIV-1 DNA sequences was present in the amplified DNA from all sources, including cloned DNA, infected cell line DNA, or fresh patient material. It is expected that the reactions were saturating, and the concentration of the final reaction products was not a reflection of the initial HIV-1 sequence concentration. In the first attempt to quantitate HIV-1 DNA sequences, we performed amplification with a variety of concentrations of cloned DNA ranging from 1 ng to 1 fg over differing numbers of cycles of amplification, 10, 15, or 20 cycles. Under these conditions, the concentration of HIV-1 sequences in the amplified products are proportional

to the initial HIV-1 DNA sequence concentrations, thus demonstrating the ability of the assay to quantitate HIV-1 DNA sequences.

In addition, we have utilized the PCR technique for the detection of HTLV-I and HTLV-II sequences from fresh patient material. Oligonucleotides were synthesized which were identical to nucleotides 7463-7486 and complementary to nucleotides 7552-7572 in the rex and tax genes of HTLV-I (14). Identical sequences are present in the HTLV-II genome (15). Amplification was performed with Taq polymerase for 30 cycles under the conditions described above using either cloned HTLV-I or HTLV-II sequences, HTLV-I infected cell lines MT2 and HUT 102, or peripheral blood or lymph node material from 6 HTLV-I infected individuals that have been referred to us. Three of these individuals had acute adult T cell leukemia/lymphoma (ATLL), one had chronic ATLL, one had an intermediate clinical syndrome, and one was asymptomatic. HTLV sequences could be detected in all samples but not from the negative controls which included the chronic myelogenous leukemia cell line K562 or the uninfected T lymphoid cell line H9. HTLV-I or HTLV-II-specific end-labeled oligonucleotide probes were utilized to distinguish in each case which HTLV sequences were present.

Conclusions

The experiments performed in the first year of this study have demonstrated a number of important findings.

1) Low concentrations of HIV-1 DNA sequences can be amplified from levels which are undetectable by Southern blot hybridization to levels which can be easily detectable.

2) The PCR reactions can be successfully performed with either E. coli DNA polymerase I or Taq polymerase. The latter DNA polymerase is advantageous since it is not inactivated during repetitive cycles of heat denaturation. Thus, using this polymerase no additions to the reaction are needed after each cycle.

3) The PCR reactions are greatly facilitated by an automated machine. This speeds up the reaction considerably, and also provides for more efficient amplification.

4) Using Klenow polymerase, we calculated the efficiency of amplification as 60% per cycle with cloned DNA. A similar amplification efficiency was estimated with the Taq polymerase.

5) The specificity of the amplification with cloned DNA was demonstrated by two different methods. Amplification was dramatically decreased by a) digestion of the target DNA with an enzyme which cuts between the primer binding sites, or b) the addition of a blocking oligonucleotide complementary to one of the two oligonucleotides used for amplification.

6) Small amounts of HIV-1 DNA sequences in cell lines infected with either of two HIV-1 isolates could be detected after PCR, whereas they could not detected in 1 microgram samples by direct Southern blot hybridization. The amplification efficiency over 20 cycles with infected cell line DNA is greater than 1000-fold. No signal was obtained using DNA from cell lines not infected with HIV-1, including HTLV-I infected cell lines.

7) Using a single set of primers, amplification could be obtained with tissue samples from 10 of 12 patients. The technique was successful with a wide range of different tissue types including brain, blood mononuclear cells, lymph node, and spleen. The technique was successful with samples in which HIV-1 DNA sequences could not be detected directly by Southern blot hybridization on the unamplified DNA. Thus, with a single set of primers, a sensitivity of over 90% was acheived. No amplified HIV-1 sequences were detected in two different tissues from each of two HIV-1 infected individuals with symptomatic disease. It is likely that in these cases, sequence heterogeneity at the primer binding sites accounted for the failure of the PCR reactions. With the use of additional primer to other conserved regions of the HIV-1 genome, it is likely that HIV-1 DNA sequences could be amplified in these samples as well.

8) HIV-1 DNA sequences could not be detected after PCR under identical conditions from any of the nine samples from uninfected individuals. This demonstrates the specificity of the method.

9) PCR allows quantitation of HIV-1 DNA sequences when performed under non-saturating reaction conditions. This was demonstrated with differing amounts of cloned HIV-1 DNA ranging from 1 ng to 1 fg of DNA, and differing number of PCR cycles, 10, 15, or 20 cycles.

10) Amplification of HTLV-I and II sequences was successful using primers to sequences which are identical in the rex/tax genes of both HTLV-I and II. HTLV sequences could be detected in fresh tissues from symptomatic and asymptomatic patients, utilizing DNA samples which are either positive or negative by Southern blot hybridization. Thus, this provides a highly sensitive method of diagnosing HTLV infection of

either type. It may be useful as a confirmatory assay or a primary screening assay. This will be increasingly important with the development of screening assays for these and other retroviruses in blood banks and particular patient populations. HTLV-I and II could also be distinguished by hybridizing these amplified products with HTLV-I or II-specific oligonucleotides. Since most antibody-based assays are unable to make this distinction, this is an important feature of this new assay which should assist in the diagnosis of HTLV infections.

Recommendations

1) The major goal of this work is to provide a routine assay to quantitate HIV-1 DNA sequences. For this purpose we plan to perform the following experiments:

a) Perform PCR reactions for 10, 15, and 20 cycles, on the fresh tissues samples from HIV-1 infected patients. This will assess the range of variation in HIV-1 DNA sequence concentrations present in the starting material. The amplified products will be analyzed by polyacrylamide gel electrophoresis, Southern blot hybridization, and densitometer analysis.

b) Develop an internal control for the efficiency of PCR utilizing globin DNA, present at the level of one copy per haploid genome.

c) Perform PCR reactions on appropriate concentrations of cloned DNAs under identical conditions as those of fresh patient material to provide a standard curve for quantitation.

2) When the above is performed successfully, we will then quantitate HIV-1 DNA sequences in fresh patient material from blood mononuclear cells or other tissues from patients at different stages of HIV-1 infection. Over the last year, we have prepared 60 such samples from untreated patients. This has been greatly facilitated by the establishment at our center of an AIDS Clinical Study Group (L. Ratner and G. Medoff, co-directors). Additional samples will continue to be obtained from both our own patients and those of other groups. We will compare the level of HIV-1 DNA sequences to the stage of disease, and the subsequent clinical course of these patients to assess the prognostic capabilities of this assay.

3) With the successful completion of the first step, we will accumulate blood samples from patients before and after treatment with anti-HIV-1 agents. We will compare changes in HIV-1 DNA sequences to the patients clinical therapeutic

responses as well as other laboratory parameters that are being collected on these patients, including p24 antigen levels and T4 lymphocyte counts. This will serve to examine the utility of this assay for testing and monitoring anti-viral agents in man.

4) We will continue to utilize the HTLV PCR amplification assay for diagnosis. This will include patients with syndromes suggestive of ATLL, as well as for screening for HTLV-I, II, or other related retroviruses in patients with T cell lymphomas, leukemias, mycosis fungoides, large granular lymphocyte leukemia, or Felty's syndrome.

Publications

Two publications from this work are in preparation:

1) Bell J and Ratner L. Specificity of the HIV-1 Primer Chain Amplification Reaction.

2) Ratner L and Poiesz BJ. Human T-lymphotropic virus type I associated leukemias in a non-endemic region.

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PCR AMPLIFICATION OLIGONUCLEOTIDES

LTR-LEADER:

GAG: