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Immunologic Intervention in HIV Infection: Anti-Polymerase Responses and Hormonal Regulation

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This work represented an integrated effort to develop novel Pol-based immunogens, and to explore mechanisms of enhancing the efficacy of both HIV immunogens and antivirals through use of peptide hormone. With the past three years of DAMD support, we have defined epitopes of HIV-1 polymerase (Pol) which correlate with serum IgG-mediated inhibition of Pol catalytic activity and limited progression of HIV disease. As Pol exhibits restricted variability and elicits potent T cell proliferative, cytotoxic and humoral responses, these regions might be considered for inclusion as candidates in peptide-based or peptide-boosted, recombinant protein-based protective and immunotherapeutic vaccines. Second, recent elucidation of certain properties of recombinant human growth hormone (rhGH) suggests direct relevance to AIDS vaccines and immunotherapeutics. rhGH markedly enhances proliferation of engrafted human CD4+ and CD8+ T cells in SCID/hu mice, and counteracts hematopoietic suppression in animals treated with AZT.
13. Abstract (continued)

It can also serve as an adjuvant, dramatically increasing the immunogenicity of a single dose of certain viral vaccines, consistent with its ability to stimulate clonal expansion of antigen-specific T cells. We have shown that rhGH can also potentiate the anti-viral efficacy of AZT, and have preliminary data addressing the mechanism and specificity of this effect.
FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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Statement of Purpose: The development of therapeutic and protective HIV vaccines is a vital issue in AIDS biology. The exploration of novel concepts, in terms of a possible HIV-1 Pol-based immunogen, based upon association of antibodies capable of inhibiting catalytic activity of Pol with long-term survivors, as well as immune enhancement, formed the basis of this proposal.

Objectives: 1. Characterization of IgG inhibitors of HIV polymerase catalytic activity with respect to HIV neutralization, viral enhancement, and reactivity to linear HIV Pol sequences.

2. Investigation of hormonal agents capable of enhancing immune responsivity to potential peptide-based vaccines.

Approach of research: 1. Evaluation of purified IgGs from HIV seropositive individuals with divergent levels of anti-Pol IgG inhibitor for HIV neutralizing antibody-dependent enhancement (ADE) activity. Preparation of overlapping 10 amino acid peptides from the entire region of Pol found to be correlated with IgG anti-Pol catalytic activity inhibitors in our prior work. Use of these synthetic peptides in Pep-Scan ELISA assays to map, in fine detail, linear epitopes correlating with the inhibitory activity. Correlation of such an ELISA with the more laborious enzymatic assay in terms of clinical prediction.

2. Preparation of EBV+ B cell lines from selected individuals with high levels of anti-Pol inhibitor, in an attempt to isolate monoclonal inhibitors.

3. Definition of mechanisms of peptide hormone enhancement of anti-peptide immune responsivity.

4. Use of reagents prepared in No. 1 to examine cytolytic T cells prepared from HIV-1 seropositive individuals with varying levels of anti-Pol IgG inhibitor.

Brief summary of accomplishments to date:

This work represented an integrated effort to develop novel Pol-based immunogens, and to explore mechanisms of enhancing the efficacy of both HIV immunogens and antivirals through use of peptide hormone. With the past 3 years of DAMD support, we have defined epitopes of HIV-1 polymerase (Pol)
which correlate with serum IgG-mediated inhibition of Pol catalytic activity and limited progression of HIV disease. As Pol exhibits restricted variability and elicits potent T cell proliferative, cytotoxic and humoral responses, these regions might be considered for inclusion as candidates in peptide-based or peptide-boosted, recombinant protein-based protective and immunotherapeutic vaccines. Second, recent elucidation of certain properties of recombinant human growth hormone (rhGH) suggests direct relevance to AIDS vaccines and immunotherapeutics. rhGH markedly enhances proliferation of engrafted human CD4+ and CD8+ T cells in SCID/hu mice, and counteracts hematopoietic suppression in animals treated with AZT. It can also serve as an adjuvant, dramatically increasing the immunogenicity of a single dose of certain viral vaccines, consistent with its ability to stimulate clonal expansion of antigen-specific T cells. We have shown that rhGH can also potentiate the anti-viral efficacy of AZT, and have preliminary data addressing the mechanism and specificity of this effect.

Specifically, we have accomplished the following:

1. 10-mers using a single amino acid overlap have been prepared from the three large domains of HIV-1 Pol which correlate best with reverse transcriptase activity (amino acids 144-191, 214-335, 511-536). These 205 overlapping decapeptides were incorporated onto pins using technology developed by Cambridge Research Biochemicals, and utilized in ELISA assays with serum samples from HIV+ individuals at various clinical stages of HIV disease. Several discrete epitopes have been identified which correlate not only with capacity to block the catalytic activity of HIV RT, but also correspond to regions identified as inhibitory using murine anti-Pol monoclonal antibodies.

2. Anti-Pol IgGs have been investigated for HIV-1 neutralization and enhancement in vitro. Direct viral neutralization does not appear to explain their activity. The ADE phenomenon was confirmed, but the level of such activity, in these specimens as well as others we’d examined, did not appear to have clinical significance.

3. In the process of generating human EBV+ B cell lines capable of secreting anti-Pol monoclonal antibodies, we attempted to directly infect purified peripheral blood B cells from HIV seronegative donors with HIV. Dr. Haseltine’s group, and others, have recently demonstrated by PCR that, in some HIV seropositive individuals,
circulating B cells may harbor provirus at levels equivalent to CD4+ T cells. We demonstrated direct infection of non-immortalized B cells by HIV, and established chronically infected B cell lines capable of responding to induction signals. This work (Laurence, et al., *Virology*, in press), represents the first model for investigation of control of HIV infection in B cells. This work also has implications for the pathogenesis of B cell lymphomas in AIDS patients, and the ability of such cells to serve as presentors of HIV as a superantigen (SAg), in SAg-mediated models of T cell anergy and apoptosis.

4. Growth hormone (somatotropin) has been used as an adjuvant in animal models to enhance B and T cell reactivity against various viral vaccines. Its ability to augment proliferation of CD4+ T cells, increase TH1-like cytokine secretion in vitro and in SCID/hu mice, and promote thymocyte development together with its anabolic properties, is of great interest in HIV disease. We have discovered that not only is growth hormone a potent T cell stimulant but it is capable of synergizing with the anti-HIV drugs/AZT and FLT in the inhibition of HIV replication. Preliminary data on the mechanism for this specific facilitation, not seen with dideoxynucleosides ddI and ddC, have been gathered.

A more comprehensive description of some of the results, together with listings of publications arising through DAMD support, is provided below.
(A) **Original articles of direct relevance to the current work:**


(B) Original articles of related relevance to the current work:


(C) Book chapters and reviews of related relevance to the current work:


**Background:** Antibodies to HIV-1 reverse transcriptase (RT), elicited by proteins associated with RNA-depende DNA polymerase and endonuclease functions, are detectable in >99% of HIV-1 seropositive individuals (1,2). They occur in serum and genital secretions (3) and, distinct from anti-core antibodies, may be independent of clinical stage, present at high titer throughout the course of HIV disease (4). Such marked, persistent immunogenicity of a Pol product has not been demonstrated for other mammalian retroviruses, including HTLV-I, in their respective hosts, and thus may have an adaptive value.

The original hypothesis of our DAMD-funded work was that generation of a subset of such immunoglobulins directed against catalytic moieties of RT would inversely correlate with clinical disease. This concept was stimulated by observations in "non-progressor" feline leukemia virus-infected cats (5). Consistent with this postulate, we demonstrated a heterogeneous immune response for inhibition of HIV-1 enzymatic activity by purified IgGs derived from HIV-1 seropositive individuals. Loss of this inhibitory phenomenon, termed RTI, preceded development of clinical disease in a small cohort studied longitudinally (1). This finding has now been confirmed in over 340 HIV seropositive individuals followed by six different groups (6-11), utilizing a variety of assays for RT function. In all reports, decline of RTI titer was prescient of clinical progression.

Our concept was highlighted at a recent NIH conference entitled "Immunologic and Host Genetic Resistance to HIV Infection and Disease" held in Washington, DC on Feb 25-27, 1993. Two points were emphasized (12), both pertaining to the theme of our work, i.e., definition of novel mechanisms for immunologic intervention in HIV disease:

a) Virtually all HIV-positive patients show an initial CD4 cell decrement from baseline to about 500/mm$^3$. In most the counts continue to decline, but in about 15% they stabilise or even rise slightly over many years. In these "non-progressors" strong cellular and humoral responses to certain highly conserved viral sequences appeared to correlate with long-term survival. The putative catalytic site of reverse transcriptase was one such epitope.

b) More attention needs to be paid to enhancing both humoral and cell-mediated immunity (CMI) in the context of protective and therapeuetic vaccinations. It was recognized that some cytokines which stimulate CMI down-regulate antibody production, and vice-versa. It was recommended that,
ideally, peptide or protein products used in active specific immunotherapy regimens should be administered along with factors that could facilitate both types of response without interfering with activity of classical antiviral nucleoside analogs.

We had approached these two mandates in the following manner:

(1) We have defined the major B-cell and concurrent B and T cell regions of Pol recognized during HIV-1 infection, utilizing overlapping peptides covering the most immunogenic portions of the molecule. We have also correlated RTI activity with sequences critical to the maintenance of RT enzymatic function. Such mapping studies are important, as MHC-restricted, CD8+ cytolytic T cells (CTL) specific for RT are found in the majority of asymptomatic HIV-seropositive individuals (13) but, unlike the discrete targets formed by highly variable envelope elements, RT CTL epitopes may be distributed throughout Pol (14). In contrast, major T helper cell recognition sites for HIV-1 RT are much more restricted (15).

(2) A problem with most protective HIV/SIV vaccines in current trials is the poor immunity elicited, particularly in terms of CTL response. Optimization of these responses, as well as reactivity to active specific immunotherapy in HIV+ individuals, would be of great value. Administration of selected HIV peptides along with HIV recombinant proteins have led to greater immunogenecity in protective vaccination strategies in macaques. Use of an immune adjuvant should also further boost these responses. With these concerns, we focused in the later part of our work on rhGH, examining its biology in terms of immune enhancement and interactions with HIV and anti-HIV nucleoside analogs.

GH receptors are present on T and B lymphocytes and monocytes, with a close structural relationship between its extracellular domain and that of a group of cytokine receptors encompassing interleukins 2,3,4,6 and 7, and granulocyte-macrophage-colony stimulating factor (GM-CSF) (16). Rodent and human T and B lymphocytes, monocytes, and natural killer cells can also secrete GH (17) or one of its primary induced products, insulin-like growth factor I (IGF-I) (18). In addition, GH can augment immunocyte proliferation, cytokine secretion, and the clonal expansion of antigen-specific T cells,
particularly with a Th1-like profile. In vivo, GH promotes generation of CD4+ and CD8+ T lymphocytes in SCID mice engrafted with human peripheral blood mononuclear cells (PBMC) (19). On this basis, rhGH has been utilized successfully to facilitate protective responses to certain viral vaccines in animals (20).

As GH is also an anabolic agent, Phase I clinical trials of rhGH have been initiated in HIV wasting syndrome (21). However, a major concern with the use of any cytokine or growth factor in AIDS is the potential for accelerating HIV replication. Indeed, in an initial report we demonstrated that pharmacologic concentrations of rhGH (>10 ng/ml) could enhance HIV replication and tumor necrosis factor-α (TNF-α) secretion in acutely infected PBMC, in the absence of viral induction from chronically infected cell lines or a direct effect on HIV-1 LTR-mediated transcription (22). Enhanced replication of HIV paralleled rhGH-mediated stimulation of CD4+ T cell proliferation and cytokine generation, and was abrogated by AZT. Unexpectedly, however, we found that rhGH potentiated the anti-viral effect of AZT, using two highly divergent but AZT sensitive isolates, TIIIB (T cell tropic) and BaL (monocytotropic). A parallel effect was obtained with AZT resistant strains. GH also increased the efficacy of a non-cross-resistant fluorinated analog of AZT, FLT (3′-fluoro-3′-deoxythymidine). No interaction was observed with two other anti-HIV nucleoside analogs, ddI and ddC. This is consistent with our observation that potentiation was associated with augmentation of a cellular dTMP kinase, leading to higher intracellular levels of the anti-RT metabolite of zidovudine, AZT-TP, in the absence of effect on phosphokinases specific for ddI. In addition, GH increased monophosphorylation of AZT, a molecule which can specifically block the RNaseH of HIV-1, another enzymatic activity essential to reverse transcription. Thus, rhGH might have value therapeutically as a vaccine adjuvant, an immune modulator, and a potentiator of AZT and FLT activity.

Specific Accomplishments, Including Data and Methodology

I. Identification of Pol epitope regions involved in IgG-mediated inhibition of RT catalytic activity.

Immune-based therapies are of great interest in HIV infection, as specific immunity is the normal means of containment and eradication of
viruses. Many approaches to preserving and restoring immune competence have been attempted, including cytokines and growth factors, immune modulators, receptor-directed therapy, and specific immune interventions with antibodies and vaccines (23). Vaccine administration is based, in part, on the premise that the prolonged course of HIV infection is attributable to specific immune responses that are initially effective but eventually fail (23). Indeed, primary immune responses during acute HIV infection lead to a $\geq 3$ log-fold decrease in viral titer, compared to a maximal $1-1.5$-log kill with AZT. This work was pioneered by Redfield, Birx, Burke, and colleagues at WRAIR using recombinant gp160 in patients in early stages of infection (24). An increase in existing anti-Env antibodies as well as appearance of specificities not detected during natural infection was elicited (24). We elected to focus on another highly immunogenic unit of HIV, Pol.

Since our original description of IgG's capable of inhibiting the catalytic activity of HIV-1 reverse transcriptases in serum from asymptomatic, "non-progessor" HIV+ individuals, there have been numerous studies confirming this effect (6-11). Loss of this inhibitory activity has been linked to declining clinical status. These antibodies presumably arise from immunizations against enzyme from circulating disrupted virions or enzyme present on the infected cell membrane, either as a mature protein or in the form of a group antigen-polymerase (Gag-Pol) polypeptide precursor. Indeed, Gag-Pol protein has been found on the surface of cells infected with mutants of murine leukemia virus blocked at late stages of virion assembly (25). It was hypothesized that certain epitopes of Pol are responsible for these inhibitory antibodies, and that such regions might serve as substrates for immunotherapy in HIV disease.

With these concerns, we have accomplished three goals. First, we have correlated humoral reactivity to six Pol peptides with RT inhibitory (RTI) activity in HIV+ individuals of known RTI status, as originally assessed by a functional assay. Second, we have correlated two of these regions with catalytic sites on Pol, mapped by murine monoclonal antibodies. Third, we have initiated a screen of some one hundred sera, including longitudinal samples, from HIV+ individuals at various clinical stages of HIV disease.

**Peptide synthesis.** Two hundred and five overlapping decapeptides, representing about 40% of Pol, including both RNA directed DNA polymerase and
RNase H domains, were used as antigens. (The Pol sequence was derived from isolate PV22, which diverges from that of the prototype HIV-1 clone BH10 at only three amino acid residues.) These regions were selected for particular scrutiny as, based upon preliminary data with large recombinant Pol polypeptides, they are highly conserved, appear to be exposed on the surface of native RT, and are immunogenic in individuals at early stages of HIV disease (26). Syntheses were performed as described by Geysen et al (27), using a PEPSCAN kit (Cambridge Res. Biochem. Wilmington, DE), and the resulting peptide products immobilized on polyethylene pins. Control peptides, PLAQ and GLAQ, were included in the synthesis, the former reacting with a monoclonal antibody (Cambridge Res. Biochem.) to ensure that coupling was completed, and the ELISA reaction capable of detecting it.

Epitope scanning assay. Polyethylene peptide pins were immersed in blocking buffer (0.5% blocking reagent (Boeringer-Mannheim), 0.1% Tween-20, 4% normal goat serum in PBS, pH 7.2) at 4°C for 16h. The pins were then incubated at 4°C overnight in microtiter plates containing 175 μl of human sera diluted 1:200 in blocking buffer. This concentration was selected based upon preliminary screen of HIV-1+ and negative sera to maximize sensitivity and specificity. Pins were then washed 4-6 times in PBS/Tween and incubated with alkaline phosphatase-conjugated affinity-purified goat-antihuman IgG, diluted 1:500 in blocking buffer, at 25°C for 1h. p-nitrophenylophosphate was used as substrate in 10% diethanolamine, 0.5 mM MgCl₂, pH 9.8. The color reaction was developed at 25°C in the dark for 1h, and evaluated in an MR600 Microplate reader at 410 nm. A positive was defined as > 3SD over control sera. Bound antibodies were removed from the pins after each assay by sonification in an ultrasonic bath (Fisher Lab Products, 200 W model) in disruption buffer (1% SDS, 0.1% 2-mercaptoethanol, 0.1 M sodium dihydrogen orthophosphate, pH 7.2) at 60°C for 45 min., followed by immersion in distilled water at 60°C, and washing in gently boiling methanol for 3 mins.

Indirect antibody ELISA. Six synthetic 10- or 11-mer polypeptides were prepared by Research Genetics, Inc. (Huntsville, AL) and used in a subsequent screen of serum samples. Their composition is provided in Table 2. Wells of 96-well polystyrene plates were coated with synthetic peptides (100 μl; 25 μg/ml in 0.1 M Tris HCl, pH 8.8), in triplicate, overnight at 4°C. The wells were then blocked with a mixture of 1% BSA and 4% normal goat serum in PBS, pH 7.2 (blocking solution). Sera were diluted 1:100 in blocking solution plus
0.5% Triton X-100, and 100μl added to antigen-coated wells. After incubation overnight at 4°C, the plates were washed with PBS plus 0.05% Tween-20. Bound human IgG was detected by adding alkaline phosphatase-conjugated goat antihuman IgG, diluted 1:500 in blocking solution. After incubation for 2h at 25°C, the wells were washed with PBS and activity assessed using a commercial kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Absorbance at 450 nm was determined in a MR600 Microplate reader. A positive represented ≥ 5SD over HIV seronegative controls.

Neutralization of RT activity. RT inhibitor (RTI) activity was determined by utilizing IgG purified from each serum by Zeta-Chrom 60 filter separation (AMF Lab products) and standard assays for reverse transcriptase (1). Enzyme activity of 100% represents [³H]thymidine monophosphate incorporation in a reaction mixture containing PBS in place of IgG. A positive result was defined as inhibition of catalytic activity by ≥ 50% with 10μg IgG.

Immunodominant RT B-cell epitope regions. Examples of typical PEPSCAN reactivity profiles for six of 17 HIV+ individuals with WR stage I through VI disease initially screened are shown in Fig. 1A-F. Several peaks could be observed with each serum, with a greater spectrum of reactivity for individuals in earlier clinical stages of disease. Reactivity of these 17 unselected HIV+ sera thereby elucidated five major antigenic Pol regions, defining one epitope region near the amino-terminal, three in the mid-portion, and one close to the C-terminus. None of the HIV negative sera recognized these sites. These have been superimposed, in Fig. 2, on a secondary structure prediction map of Pol, prepared by computer-generated algorithms defining potential B and T units. They represent areas of amino acid sequence stability among geographically disparate HIV-I isolates (28). Epitope Region I encompasses an α-helix rich region predicted to have moderate B and T cell reactivity, Epitope Region II, III, and IV fit strong B-cell segments, and Epitope Region V overlaps a B-cell segment of moderate predicted strength. Frequencies of recognition of these initial five regions are given in Table 1, together with indication of the presence of RTI. Reactivity of Epitope Region I was correlated with this inhibitor (p = 0.01; Fisher exact test (two-tail)), while most sera (15/17; 88.2%) recognized Epitope Region III, regardless of clinical stage or RTI titer.

Pol peptide reactivity and advancing clinical disease. In a preliminary
attempt to refine the portions of these five regions most closely linked to antibodies capable of mediating neutralization of RT catalytic activity, serial serum samples from six individuals at various clinical stages of HIV disease, previously known to have altered their RTI capacity with time, were evaluated by PEPSCAN. Three examples from these matched pairs, representing individuals who had not been treated with an anti-HIV nucleoside analog, are presented in Fig. 3. In agreement with the data in Table I utilizing unselected sera, reactivity to Epitope Regions III and V remained stable despite advancing clinical disease and loss of RTI, while serologic reactivity within regions I and II, declined with loss of RTI capacity.

Correlation of linear Pol regions linked to RTI activity by PEPSCAN with B-cell epitope regions mapped by anti-Pol monoclonal antibodies. Several groups have generated murine monoclonal antibodies to recombinant HIV-1 RT, permitting identification of multiple antigenic sites on HIV-1 Pol. A subset with RT neutralizing capacity is summarized in Fig. 4. In particular, Szilvay et al (29) noted RTI with two monoclonal antibodies. One mapped to aa 193-284 of Pol, adjacent to a sequence, YMDD, strictly conserved in a region of nonpolar amino acids involved in polymerase-template binding (30). The importance of this sequence was confirmed by another report, linking RT monoclonal antibody-mediated inhibition to aa 200-230 (31). The aa 294-319 region was also shown to mediate RTI by three studies, involving monoclonal antibodies recognizing aa 294-319 (20), 231-353 (32), and 294-302 (33). A rabbit anti-Pol antiserum identified a final domain linked to RTI, mapping to the COOH terminus at aa 536-549 (34). Notable symmetry between these regions and B-cell epitope regions recognized by our peptide scanning data is apparent by comparison of Figures 2 and 4. This is important, as so far we lack data on asymptomatic HIV seronegative individuals immunized with Pol-containing immunogens. This would provide information on B cell sites recognized in the face of full T cell help, and may be forth coming from trials of the Salk HIV immunogen. However, the overlap we demonstrate between RTI epitopes mapped by HIV+ individuals and immunized mice suggests that the epitope regions identified by sera from HIV+ asymptomatic individuals might be expected to mimic those in immunocompetent humans.

Definition of specific Pol epitope regions linked to RTI in an extended patient population. The patterns of decapeptide recognition by sera from unselected and paired samples were compared with the presence or absence of
RTI. Those corresponding to regions critical for RT function, based upon monoclonal antibody-mediated RTI assays described above, were given special attention. We discriminated among several possible sequences described by PEPSCAN by requiring reactivity with at least three consecutive pins.

This led to a final selection of six synthetic peptides, either 11- or 12-mers, to screen for reactivity against 57 HIV-1+ sera. As can be seen from Table 2, peptide 5 was the most immunogenic, recognized by 15/18 stage I individuals and 12/39 CDC clinical in stages III, IV. When only those sera capable of reacting to any of the six peptides were evaluated, this result was more prominent, identifying 15/16 stage II and 12/12 advanced patients. This finding was anticipated by our PEPSCAN data, as peptide 5 (aa 257-266) is part of the highly immunogenic Epitope Region II (aa 244-269). In addition, a statistically significant association between absence of reactivity to peptides 1 and 2, representing portions of Epitope Region I, and advanced clinical stage of HIV disease was obtained. For individuals reacting with any Pol peptide, peptide 1 was positive in 13/16 stage II, but only 2/12 stage III, IV (p<0.002), while peptide 2 was positive in 14/16 stage II but in 4/12 stage III, IV (p<0.005).

Finally, it appears that these differences truly represented a loss of specific responses to some Pol segments rather than a general decrease in titer to all peptides. Six sera were selected for dilutional analysis with reactivity maintained to peptide 5 in the face of loss of reactivity to peptide 1 and/or 2 at serum dilutions > 1:1000.

II. Potential use of Pol epitope regions in screening for emerging HIV-related pathogens.

An unanticipated benefit of our Pol peptide scanning data may be its potential as a preliminary screen for lymphocytopathic retroviruses in individuals with a newly described syndrome, idiopathic CD4+ T lymphocytopenia (ICL). A few Pol regions are extraordinarily well conserved throughout all known mammalian retroviruses, and certain of these appear to be highly immunogenic. We have utilized synthetic peptides prepared from these sequences to screen sera from individuals at high risk for a potential retroviral etiology for their immune deficiency. That is, we are interested in identifying patients who present with clinical AIDS or ARC, in the absence of HIV-1,-2 infection or hypogammaglobulinemia, together with CD4:CD8 ratios
<1 and evidence for a persistent, progressive decline in CD4 count to < 300/mm³. We believe that these features will distinguish patients with immune deficiencies that are novel, acquired, and may require opportunistic infection prophylaxis, and not simply reflective of underlying confounding factors related to either "physiologic" CD4 lymphopenia, well-known infections which may transiently alter CD4 counts, congenital disease, etc. We have identified two such individuals from over 20 cases meeting the CDC definition of ICL at our hospital, both exhibiting reverse transcriptase activity in culture samples in the absence of evidence for HIV-1,2 infection (35), and have already screened two individuals identified through a WRAIR program on ICL. Our concept, a review of the literature, has been published in the Annals of Internal Medicine (36).

III. Effect of rhGH on cellular proliferation, cytokine generation, and acute HIV infection.

rhGH (Bio-Tropin), free of endotoxin, was obtained from Bio-Technology General (New York, NY) and prepared as a stock solution of 5 mg/ml in distilled water. Physiologically it is the product of the GH-N gene on human chromosome 17, primarily expressed in the anterior pituitary, and comprises the 191 amino acid, 22kd molecule generally referred to as somatotropin. Typical pharmacologic serum levels are in the range of 10-20 ng/ml. We initially found that peripheral T cell proliferation was enhanced by 5-50 ng/ml rhGH at all time points assessed, in the presence or absence of HIV infection (22). As rhGH can activate cytokine secretion, we also examined its effect on production of IL-6 and TNF-α, both linked to HIV activation on transcriptional and post transcriptional levels. They were assessed by ELISA (R & D Systems, Minn., MN). There was no change in IL-6, which remained in the range of 60 pg/ml, similar to that seen by others following HIV infection of mitogen-activated PBMC. In contrast, levels of TNF-α increased 3-4 fold over control, HIV infected cultures in the presence of > 10 ng/ml rhGH (17 ± 1 pg/ml vs. 64-70 pg/ml). These results were consistent with our finding of an increase in viral production in acutely infected PBMCs exposed to pharmacologic concentrations of rhGH (22). This is an important concern as one of six patients became HIV p24 antigenemic within six weeks of entering a phase I trial of rhGH in HIV-associated cachexia (21). It must also be assessed in the context of a recent study showing significant increases in p24 antigenemia.
among five of six HIV-seropositive individuals treated with another immune growth factor, GM-CSF.

Such adverse results could severely limit the feasibility of immune modulators to potentiate HIV vaccine responses. However, we now have evidence that broad concentration ranges of rhGH, including levels not linked to upregulation of HIV replication in vitro, can potentiate the anti-viral activity of two dideoxynucleosides, AZT and FLT, in the absence of interaction with two related compounds, ddI and ddC.

**Effects of rhGH on dideoxynucleoside analog inhibition of HIV-1 replication.**

The inhibitory effects of AZT and its fluorinated analog, FLT, on HIV-1/TIIIIB replication have been exhaustively documented in vitro using a variety of methodologies. IC₅₀’s, determined by either reverse transcriptase or p24 Gag assay, range from 0.006 μM in MT4 cells to 0.05 μM in H9 cells with AZT, and 0.005μM in MT4 and 0.017 μM in H9 cells with FLT. These data were replicated for AZT utilizing PBMC and HIV-1 strain TIIIIB, with an IC₅₀ to 0.0035 μM (Fig. 5). Despite its enhancement of HIV replication, a high concentration (250 ng/ml) of rhGH facilitated the activity of ACT, lowering the IC₅₀ to 0.0035 μM. Even more striking was the ability of a moderate dose (10 ng/ml) of rhGH to similarly potentiate AZT, decreasing the IC₅₀ by up to 100-fold (Fig. 5). A parallel, if less potent effect was noted for AZT-mediated inhibition of BaL replication, with a >10-fold decrease in IC₅₀ in the presence of 10 ng/ml. The antiviral activity of the fluorinated AZT derivative FLT was similarly potentiated by rhGH when tested against the sensitive isolate TIIIIB.

These alterations in the magnitude of viral suppression were not linked to a non-specific or toxic effect of dideoxynucleoside analogs in the presence of GH, as shown by two methods. First, total numbers of recoverable viable cells and DNA synthetic responses were not depressed by addition of rhGH to the concentrations of AZT or ddI used. Second, such potentiation was not recognized utilizing ddC or ddI (Fig. 6). Indeed, in some experiments 10-fold higher concentrations of ddC (Fig. 6A) or ddI (Fig. 6B) were required to inhibit replication in the presence of 10 or 250 ng/ml rhGH, as compared with control cultures inoculated with HIV TIIIIB in the presence of buffer.

**Interaction of rhGH and AZT with AZT-resistant isolates of HIV-1.** Increases in IC₅₀ to AZT among patients manifesting clinical progression on such therapy have been linked to four predominant mutations in HIV-1 pol. While the clinical significance of such resistance is unclear, the temporal association
between recognition of this phenomenon and development of either advancing clinical disease or progressive decline in CD4 count has prompted the search for agents which can abrogate this effect. Utilizing one clinical isolate (G-910-6) obtained from Dr. D. Richman, exhibiting high levels of AZT resistance, we found that the IC₅₀'s were similar, but IC₇₅, IC₉₀, and IC₉₅ were progressively reduced up to 63-fold in the presence of 10-50 ng/ml rhGH.

Metabolism of AZT and ddI in the presence and absence of rhGH. There is no evidence that HIV can induce unique nucleoside-metabolizing enzymes. Instead, treatment of cells with 2', 3'-dideoxypurine and pyrimidine nucleosides leads to accumulation of the nucleoside and its phosphorylated derivatives into acid-soluble and acid-insoluble material in a dose-dependent manner, independent of the presence of HIV. We thus investigated the effect of rhGH in mock infected cultures on the metabolism of AZT and ddI to their mono-, di-, and tri-phosphorylated moieties. These procedures were conducted as previously described (37). Briefly, PHA-stimulated and unstimulated PBMC, mock infected, were incubated with 1.0 μM [³H]AZT or [³H]ddI at 37°C for different time intervals. Cells were harvested, extensively washed with PBS, and viability determined. Trichloracetic acid cell extracts were then prepared, neutralized with tri-n-octylamine in trichlorotrifluoroethan (Freon), and subjected to HPLC analysis by anion exchange in a Partisphere SAX-5 column (Whatman, 12.5 x 0.4 cm). This involved a linear gradient of 0.02 to 0.7M KH₂PO₄ (pH 3.5) at 45°C, developed over 52 min. at a flow rate of 2 ml/min., with monitoring of absorbance at 260nm. Mono-, di-, and triphosphate levels were expressed as the amount of nucleotide in picomoles per 10⁶ viable cells. For all analyses, the elution times of radioactive peaks were determined by comparison with authentic standards. As shown in Table 3, mono-, di-, and tri-phosphorylated forms of AZT were all significantly elevated in the presence of hormone, with peak efficacy at 10 ng/ml of GH, while no change was noted for ddI.

Future Plans
The high immunogenicity of HIV pol gene products, as determined by binding studies, is remarkable. The presence of antibodies recognizing these proteins on immunoblot appears to be independent of clinical status. In contrast, a functional inhibition of HIV associated polymerase activity is associated with the IgG fraction of certain HIV seropositive sera and linked with serologic
reactivity to defined Pol regions. This activity appears to be lost in many asymptomatic individuals prior to the development of an HIV-associated clinical disorder. We will continue to probe the mechanism of this apparent association and explore the possibility of its utility in immunotherapeutic schemes and possibly in primary vaccinations, in association with whole recombinant Gag, Pol and Env products.

(1). Assay of humoral reactivity to 6 selected Pol peptides in a survey of longitudinal samples of HIV-1+ sera, paired with clinical data, western blot pattern, and CD4 counts, will be pursued and correlated with prolonged asymptomatic intervals. While we were fortunate to have collected a large battery of sera from individuals at all stages of HIV infection prior to the use of anti-virals, these early sera were not paired with CD4 values. It will be particularly important to determine whether this assay offers any additional independent information on clinical course when compared with carefully obtained, serial CD4 counts. The large database and stored sera collected through the U.S. Army Natural History cohort (38) would be invaluable for this study in an attempt to confirm that clinical correlations seen with RTI enzymatic assay continue to hold for our serologic assay. In addition, we have received a battery of coded sera from Dr. Sheldon Landesman of Downstate Medical Center, representing paired mother-infant specimens, for western blot testing. Controversy exists over whether those infants born to HIV+ mothers who escape infection bear maternal IgG recognizing V3 loop peptides of gp120 (39). We could evaluate these specimens for correlation of anti-Pol reactivity and inhibition of HIV transmission. This should be completed in the first year.

Clearly, peptide fragments providing linear or "continuous" epitopes may not accurately mimic the complete structure of the native determinant. This is best approximated, however, when antibodies to such fragments are able to neutralize a biologic activity associated with the native protein, as is the case with RT. This forms part of the basis for our confidence that our serologic assay will continue to show the clinical correlations exhibited for RTI by our group (1) and many others worldwide (6-11). Serial serum dilutions (1:50 to 1:1600) will be utilized to examine whether reactivity to specific Pol peptides has been altered, rather than a general decline in antibody titer. Statistical analysis for our original study was performed by Dr. Eric
Jaffe of our Division, who has been a statistical consultant for Division projects, and will continue to serve in this capacity.

(2). Correlation of anti-RT epitopes identified serologically with anti-RT cytotoxic T cell responses. The mechanism by which RTI correlates with stable clinical disease remains unclear. As noted above, group antigen-polymerase polypeptide precursors may be exposed on the surface of retrovirus infected cells, and antibody to such molecules can interfere with virion assembly or budding. In addition, RTI may be a surrogate marker for other protective cellular immune responses. The 2 of 6 immunogenic Pol regions we had identified as correlating, in a highly statistically significant manner, serologic reactivity with functional inhibitory activity to RT are putative T cell epitopes (Table 4). We will thus follow up on the observations of several groups (13, 14) suggesting that Pol is a strong target for cytolytic T cells in asymptomatic HIV infected individuals. This had originally been planned as part of our previous proposal, but as it was cut from a planned 5 to 3 year project, with a decrease in funds, we have accumulated the accessory cells and pol constructs but have not yet conducted the actual experiments.

We have prepared and stored at -135°C spontaneously immortalized EBV+ B lymphoblastoid cells lines from several of our patients. These can be infected with the Moss VAC/pol construct (13), or transfected with truncated pol genes. The latter represent seven HIV-1 pol constructs, all cloned in pBM103 and inserted in E. coli HB101. They were provided by Dr. Sean Nowlan, and encompass our five Pol epitope regions. This should be completed by the second year. We will also attempt to use the soluble peptides themselves as targets with autologous B cells. This latter system is less certain, however, as appropriate peptide folding required for MHC interaction may not occur with soluble peptides.

(3). Immune enhancement of CTL precursors by rhGH. A few tantalizing reports address the potential in vivo efficacy of HIV Pol-specific CTL. For example, CTL precursors for HIV Pol (and Env) were detected during acute symptomatic HIV infection, 11 weeks prior to antibodies capable of neutralizing the patient's own HIV-1 isolate (40). Rises in CTL precursor frequency correlated with reduction in viral load. In addition, early in the course of HIV disease the bulk of HIV replication appears to occur in lymph nodes. It has been
recently shown in a murine system that certain short peptides can rapidly induce specific CTL in lymph nodes (41). However, these responses are brief and may not represent the full repertoire of possible anti-Pol CTL responses. rhGH should serve as a potent adjuvant for such reactivity, either administered simultaneously with a primary protective vaccine, or to boost immune responsivity to therapeutic peptide or polypeptide vaccines. If this concept continues to appear feasible, it may also suggest the use of therapeutic vaccines in individuals with much lower CD4 counts than have been heretofore treated.

II. Ancillary projects related to Pol-epitope mapping and rhGH.

(1) WRAIR investigators are studying the magnitude and etiology of absolute CD4 count variation in normal and HIV-infected adult males. They concluded that it could be explained, in part, by diurnal cycle (42), and speculations as to the role of cortisol were raised. We believe that determinations of plasma GH and its major induced product, IGF-I, may provide a partial mechanism for the CD4 circadian pattern. As shown in Fig. 7, GH levels are at their nadir during troughs in CD4 cycle, with elevations preceding rises in CD4. We could assist in evaluating this possible link between CD4 values and circulating IGF-I (a more sensitive assay for GH) and induced GH, utilizing our radioimmunoassay systems.

(2) The issue of CD4 standardization and variability is also important in assessing the significance of idiopathic CD4+ lymphocytopenia (ICL). Diagnosis is predicated on a single immune parameter, a CD4 count <300/mm3, subject to multiple analytic and biologic variances. The same phenomenon is referred to as severe unexplained HIV seronegative immune suppression (SUHIS) by the World Health Organization, with the additional requirement of a disease indicative of a cellular immune deficiency. The latter raises a further concern, as infections pathognomonic of AIDS may directly influence production or compartmentalization of T cell subpopulations. Such confoundings ensure that ICL/SUHIS represents a myriad of phenomena with diverse etiologies, from "physiologic" CD4 lymphopenia to late manifestations of congenital immune defects, etc.

This is not surprising. In one large study (43), 0.6% of HIV negative controls had combined changes of CD4 lymphopenia and an abnormal CD4:CD8 ratio.
(<1.0). These were stable values, however, including several people with CD4 counts <300/mm³ who were followed for five years. Yet, it has been predicted that if the CDC definition of ICL were widely used, over 100,000 ostensibly healthy individuals in the U.S. alone might qualify.

Indeed, in virtually all of the few ICL patients recently investigated by reverse transcriptase measurements in viral cultures at the CDC, no evidence for retroviral activity was found. However, these individuals did not not show progressive decline in CD4 counts, and were thus accurately described as not having an "HIV-like" immunologic picture. The failure to detect a lymphocytopathic microorganism or retrovirus should not discourage or deflect a thorough analysis of that subset of ICL/SUHIS patients with T cell changes more characteristic of HIV disease. We hypothesize that seroreactivity to certain of our Pol epitopes may assist in a screen for HIV-1 or related retroviruses in HIV seronegative individuals and are cooperating with a group in the Division of Retrovirology to perform these assays on selected patients. We have two ICL patients with AIDS-like illnesses that have such reactivity. In a search for HIV-related sequences, we are utilizing degenerate, nested pol primers prepared from these Pol sequences. In addition, we are collaborating with Dr. M. Clerici of Dr. G. Shearer's group at the NCI. He has developed an assay for HIV-specific Env and Pol helper T cell activity in human PBMCs, and found that such responses precede detection of HIV infection by PCR and serology (44). In addition, it may detect evidence for exposure to HIV antigen or defective virus in individuals percutaneously inoculated with HIV contaminated material.
In the background control values subtracted, are placed vertically.

Figure 1: HIV-1 epitope scanning data for six representative HIV-1+ sera. Overlapping decapeptides covering the
Figure 2. Major B-cell epitopes of HIV-1 Pol, superimposed on an antigenic determinant/secondary structure map derived by Sternberg, et al. (31). Epitopes I through V, identified by PEPSOAN screening of unselected HIV-1+ sera and running in order (top to bottom), are highlighted. SS-PRED: secondary structure prediction; V, variable; G, gap; αH, α-helix; βS, β-sheet; +, strong epitope; -, moderate epitope; numeral between signs, rank order of hydrophilic peaks, as per the hydrophilicity profile of Hopp and Woods.
Table 1. HIV-1 Pol Immunodominant B-cell epitope regions

<table>
<thead>
<tr>
<th>Epitope Region</th>
<th>Amino acid Sequence</th>
<th>aa position</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>QYNVLPQGKWGSPAIFQSSMTKILEPFRKQNP</td>
<td>145-177</td>
<td>7/8 1/8</td>
</tr>
<tr>
<td>II</td>
<td>TPDKKHQKEPP</td>
<td>216-226</td>
<td>3/3 0/3</td>
</tr>
<tr>
<td>III</td>
<td>IVLPEKDSWTNVKIQKLVGKLNWASQ</td>
<td>244-269</td>
<td>8/15 7/15</td>
</tr>
<tr>
<td>IV</td>
<td>LTEEAELELA</td>
<td>295-304</td>
<td>8/15 7/15</td>
</tr>
<tr>
<td>V</td>
<td>IIEQILIKKEKV</td>
<td>521-531</td>
<td>4/9 5/9</td>
</tr>
</tbody>
</table>

*aAmino acid sequence and position number after Ratner et al., 1985.

bRT inhibitor determined as described in text. Differences between RTI+ and RTI- patients were significant only for epitope I (p=0.01).

Table 2. Correlation of anti-HIV-1 Pol epitope region reactivities with clinical disease

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>aa position</th>
<th>Reactivity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TOTAL NO.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CDC Stage</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>1</td>
<td>PAIFQSSMTKI</td>
<td>157-167</td>
<td>13/18</td>
</tr>
<tr>
<td>2</td>
<td>LEPFRKQMPDI</td>
<td>168-178</td>
<td>14/18</td>
</tr>
<tr>
<td>3</td>
<td>TPDKKHQKEPP</td>
<td>216-226</td>
<td>2/7</td>
</tr>
<tr>
<td>4</td>
<td>IVLPEKDSWT</td>
<td>244-253</td>
<td>11/18</td>
</tr>
<tr>
<td>5</td>
<td>IQKLVGKLNW</td>
<td>257-266</td>
<td>15/18</td>
</tr>
<tr>
<td>6</td>
<td>LTEEAELELA</td>
<td>295-304</td>
<td>12/18</td>
</tr>
</tbody>
</table>

*aAmino acid sequence and position number after Ratner et al., 1985.

bBy Fisher Exact Test (two-tail), reactivity with peptide 1 (p=0.002) and 2 (p=0.005) was highly significantly associated with asymptomatic disease. There was no significant difference for the other peptides.
Figure 3. HIV-1 RT epitope scanning data for three representative longitudinal samples of individuals progressing with HIV disease. The assay and axis designations are defined in the legend to Fig.1.
Patient 1: A, sample obtained within two months of an acute seroconversion reaction; B, 2 years later, stable, generalized lymphadenopathy.
Patient 2: A, asymptomatic, CD4 200/mm³; B, 2 years later, AIDS.
Patient 16: A, asymptomatic, CD4 > 500/mm³; B, 3 years la' CD4 250-300/mm³, recurrent vaginal candidiasis.
Figure 4. Regions of HIV-1 Pol linked to mAb-mediated RT inhibition. Data based on murine mAb RT blocking studies have been superimposed on an antigenic determinant/secondary structure map derived by Sternberg, et al. See legend to Fig. 2 for explanatory notes.
Figure 5. HIV-1 suppression by AZT in the presence or absence of rhGH. 500 TCID-50 of HIV-1/TIIIB (left panel) or 100 TCID-50 of HIV-1/BaL (right panel) were used to infect PHA-stimulated PBMC on day 0 in the presence of buffer (open circles), 10ng/ml rhGH (closed circles), or 250ng/ml rhGH (closed squares). Viral replication was assessed by p24 Gag antigen capture ELISA.

Figure 6. Effect of ddc and ddi on HIV-1/TIIIB replication in the presence and absence of GH. The effect of rhGH, 10ng/ml (closed circles) or 250ng/ml (closed squares) on the ability of ddc or ddi to inhibit HIV replication was compared to buffer (open circles). Assays were established as described in the legend to Fig. 5.
Table 3. Formation of [3H]AZT metabolites in PBMC in the Presence and Absence of Acute rhGH Exposure

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Total AZT Nucleotides (p mole/10^6 cells)</th>
<th>Phosphorylated Nucleoside Monophosphates (p mole/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.47 ± 0.81</td>
<td>11.93 ± 0.87 0.28 ± 0.07 0.26 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>22.78 ± 4.93</td>
<td>21.85 ± 4.65 0.62 ± 0.33 0.46 ± 0.05 &lt;0.05</td>
</tr>
<tr>
<td>50</td>
<td>19.35 ± 1.80</td>
<td>18.55 ± 1.56 0.46 ± 0.15 0.34 ± 0.01 &lt;0.05</td>
</tr>
</tbody>
</table>

PBMC (2x10^6/ml), pre-activated for 48h with PHA (5μg/ml), were incubated for 12h with 1μl of [3H]AZT in the absence and presence of various concentrations of rhGH. Cells were washed three times with prewarmed medium, and neutralized TCA extracts analyzed with anionic exchange HPLC using a Partisphere SAX-5 column.

Figure 7. Circadian rhythms for GH and cortisol in a human subject.

References


33. Örvell C, Unge T, Bhikhabhai R, Bäckbro K, Rudén U, Strandberg B, Wahren B, Fenyo EM. Immunological characterization of the human immunodeficiency virus type 1 reverse transcriptase protein by the use


