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EXPRESSION OF INTERLEUKIN-10 *IN VITRO* AND *IN VIVO*

A thesis submitted to the School

of Graduate Studies

University of Ottawa

In partial fulfilment of the requirements for the degree of

Master of Science

Department of Microbiology and Immunology

Faculty of Medicine

By

Sepideh Karimi

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ABSTRACT

Classification of cytokines into T helper type 1 (Th1) and T helper type 2 (Th2) has helped in the elucidation of the mechanisms of resistance or susceptibility to infections. HIV infection causes $CD4^+$ T cell dysfunction and depletion by indirect mechanisms; for example: inhibition of immunoregulatory cytokines. Interleukin (IL-10), the subject of this study, is secreted mostly by $CD4^+$ human Th2-like, but also by Th0,Th1-like, and by a proportion of $CD8^+$ T cell clones. HIV sero-positive patients exhibit depressed cell mediated immune responses, B cell hyperplasia, and hypergammaglobulinemia which may result from downregulation of Th1 and upregulation of Th2 class responses respectively. In this study, the expression of interferonγ (IFNγ) and IL-10 which mediate Th1 and Th2 responses respectively, in unstimulated peripheral blood lymphocytes (PBLs) from HIV^+ patients were investigated. IL-10 expression as observed by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis was significantly upregulated in patients with less than 400 $CD4^+$ T cells in comparison with HIV^+ patients with more than 400 $CD4^+$ T cells and normal controls. A semi-quantitative RT-PCR analysis of unstimulated PBLs further demonstrated IL-10 upregulation was inversely associated with IFNγ downregulation in the same individuals. Similar results were observed as determined by measuring IL-10 and IFNγ production in the supernatants of unstimulated PBLs by employing enzyme immunoassay techniques. These results suggest that HIV infected individuals express predominately Th2 type cytokines in their PBLs.

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I would like to dedicate my thesis to my father, who has taught me dignity and courage; to my mother, who has taught me love and patience; and to my brother, who has taught me sincerity and devotion.

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LIST OF ABBREVIATIONS

AIDS	Acquired immuno-deficiency syndrome
Ab	Antibody
APC	Antigen-presenting cell
BCRF-1	BamHI fragment C rightward reading frame number 1
°C	Degree Celsius
CMIR	Cell-mediated immune response
CSIF	Cytokine synthesis inhibitory factor
DTH	Delayed type hypersensitivity
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic Acid
hIL-10	Human interleukin-10
HIV	Human immunodeficiency virus
IFNγ	Interfronγ
IL-10	Interleukin-10
IL-12	Interleukin-12
LNC	Lymph node cells
mAB	Monoclonal antibody
MDF	Macrophage deactivating factor
MHC	Major histocompatibility complex

mIL-10	Mouse interleukin-10
mM	Milli-molar
NK	Natural killer cell
NKSF	Natural killer cell stimulatory factor
N	Normal
PBL	Peripheral blood lymphocytes
PBS	Phosphate saline solution
PHA-M	Phytohemagglutinin M-form
SF9	*Spodoptera frugiperda* cell line
Th	T helper
TGF	Transforming growth factor
TNF	Tumour necrosis factor
vIL-10	Viral interleukin-10

CHAPTER 1

Introduction

The principal function of the immune system is to protect animals from infectious organisms and their toxic products. The immune system has evolved a powerful range of mechanisms to locate foreign cells including; bacteria, viruses, fungi, protozoa and any other macromolecules, even body's own cells in the case of autoimmune diseases, to neutralize such invaders and to eliminate them from the body (Harlow, 1988). There are two classes of immune response: 1) humoral and 2) cellular (Roitt, 1990).

The class of immune response is specific for the profile of cytokines that are induced and for the antigen against which they are triggered. Humoral and cell-mediated immune responses can be mutually exclusive (Parish, 1972; Katsura, 1977). The ability of an organism to deal effectively with an antigen depends on which class of immune response is triggered. For example, delayed type hypersensitivity (DTH), a cell-mediated immune response is produced in C57BL/6 mice by the parasite *Leishmania major*, resulting in resolution of the infection. In comparison, BALB/c mice mount a humoral immune response after infection with this parasite resulting in an overwhelming infection and death (Howard, 1980a; Howard, 1980b). These two types of immune responses to *Leishmania* are also seen in human infection (Carvalho, 1985; Badaro, 1986; Sacks,

1987). Another human disease which shows similar characteristics is leprosy. Tuberculoid lesions in leprosy are accompanied by a strong DTH response that ultimately kills the micro-organisms and clears the infection, while in lepromatous leprosy, there is a humoral response and a weak cell-mediated immunity response, which allows the organism to multiply and the disease to persist (Bloom, 1984a; Bloom 1986b).

Th1 AND Th2 IMMUNE RESPONSES

Studies of cytokine production by mouse T helper (Th) cell clones have shown direct evidence for the existence of at least two different Th subsets (Th1 and Th2) which may have a common precursor (Th0). The Th1 cell subset is preferentially involved in cell-mediated immunity and Th2 cells are primarily responsible for the regulation and support of B cell or humoral responses (Mosmann, 1987; Coffman, 1988; Mosmann, 1989; Street, 1991). Th1 clones secrete IL-2, IFNγ, and lymphotoxin (LT). Th2 clones secrete IL-4, IL-5, IL-6, and IL-10. Th1 clones are involved in cell mediated immune responses. The presence of these two types of Th cells could explain the mutually exclusive nature of cellular and humoral immune responses. IFNγ, a Th1 product, inhibits proliferation of Th2 clones in vitro, and IL-10, a Th2 product, inhibits IFNγ production by Th1 cells (Howard, 1992c). Th0 cells may secrete Th1- and Th2-type cytokines. T helper lymphocytes serve two major functions in the induction of antibody responses to protein antigen. First, the cognate interaction of helper cells with antigen-presenting B cells delivers a signal that is undefined biochemically but may be essential for initiating B cell growth and/or differentiation. Second, the T cells secrete cytokines that stimulate

B cell proliferation and antibody secretion (Vitetta, 1989; Abbas, 1988). Some observations suggest that the nature and magnitude of the antibody response are determined by three major factors: a) the type of cytokines secreted by the T cells, b) whether the B cells are involved in cognate interactions with the helper T cells or are bystanders at the site of T cell stimulation, and c) the prior state of activation of the B cells (Abbas, 1990).

The existence of T helper cells with different phenotypes, which could determine the function of the two arms of the immune response, was first suggested in mouse model studies. The mouse helper T-cell clones differ in their effector functions and cytokine secretion patterns. If the function of Th1 and Th2 clones reflect the different classes of immune response, then these cells may be mutually inhibitory (Fernandez, 1988; Gajewski, 1988; Horowitz, 1986). Th1 cells help B cells in a linked, antigen-specific manner, are "monogamous" in limiting dilution cultures, and are required early in the immune response. These T cells may also help in an unlinked manner at high antigen concentrations (Asano, 1982). In contrast, Th2 cells help B cells in a non-linked manner, are "polygamous" in limiting dilution cultures, and are required later in the immune response (Mosmann, 1986).

INTERLEUKIN 10

IL-10 is a recently described cytokine produced mostly by T cells (Moore.1990). The molecular weight of IL-10 is 18.7 kDa . The mature human IL-10 protein is 160 amino acids in length and contains two potential sites for glycosylation, five Cys residues,

and nine Met residues. Although IL-10 was first described as a cytokine synthesis inhibitory factor (CSIF), subsequent studies have revealed its pleiotropic activities (Moore, 1990; MacNeil, 1990; Go, 1990). IL-10 is also produced by B cells and may account for the novel mast cell stimulatory activity detected in their supernatants (O'Garra, 1990). IL-10 is a potent coregulator of mast cell growth (Thompson-Snipes, 1991). Different studies indicate that the optimal growth of mast cells is dependent on multiple factor interactions. Identification of such cytokine networks may help explain the profound regulatory effects that T cells exert on this lineage during certain allergic and inflammatory responses (Galli, 1990; Rennick, 1985; Smith, 1986; Rennick, 1987; Hulter, 1990).

IL-10 suppresses Th1 cells only in the presence of accessory cells, particularly macrophages. The effect of IL-10 on macrophages overlaps with, but is distinct from the effects of two previously described cytokines that suppress the function of mouse macrophages, transforming growth factor β (TGF-β) and macrophage deactivation factor (MDF). It has been shown using neutralizing antibodies that all three macrophage suppressor factors seem to act independently (Bogdan, 1991; Fiorentino, 1991). In the mouse model, IL-10 (mIL-10) or cytokine synthesis inhibitory factor (CSIF) is produced by Th2 cells and inhibits the synthesis of cytokines, especially that of IFNγ, by Th1, but not by Th2 cells (Fiorentino, 1989). This inhibition of cytokine secretion by IL-10 has been found to be indirect and requires the presence of antigen presenting cells (APC). Also, it was shown that cytokine production by Th1 clones was affected only when macrophages, and not purified conventional cells or LY1+ B cells, were used as APC. It

has been shown that IL-10 has multiple biological activities. IL-10 has mast cell growth factor activity in combination with IL-3 and/or IL-4, and T cell growth factor activity on mature and immature mouse thymocytes when cultured in the presence of IL-2 and IL-4. Also, IL-10 increases the expression of major histocompatibility complex MHC class II antigens on small murine splenic B cells and enhances the viability of these cells (Fiorentino, 1991; Thompson-Snipes, 1991; MacNeil, 1990; Go, 1990). In addition, mIL-10 was shown to be produced by murine LY1+ B cell lymphomas and may act as an autocrine growth factor for these cells (O'Garra, 1990).

Th1 AND Th2 IN HUMANS

Human T-cell clones do not display the distinct Th1/Th2 patterns observed for murine clones. Human diseases, however, do show the corresponding differences in the class of immune response (Vieira, 1991). IL-10 is able to enhance MHC class II expression on B cells, and it inhibits MHC class II expression on monocytes. It has been suggested that the effect of IL-10 on monocytes is distinct from the inhibition of MHC class II molecules since reversal of the IL-10-induced inhibition of MHC class II expression on monocytes by IL-4 did not reverse the effect of IL-10 on T cell proliferation (Enk, 1992).

IL-10 inhibits lymphocyte the production of IFNγ by T and NK cells, and also prevents proliferation of T cells (Fiorentino, 1991; Hsu, 1992; Taga, 1992). IL-10 is a potent inhibitor of monocyte/macrophage functions, including oxidative burst, nitric oxide production, cytotoxicity, and production of cytokines, such as TNF-α and IL-1 (Bogdan,

1991; Gazzinelli, 1992; de Waal Malefyt, 1991; Ralph, 1992; Fiorentino, 1991; Oswald, 1992). TNF-α is an important cofactor for the induction of IFNγ production by murine T and NK cells and it has been suggested that the ability of IL-10 to prevent TNF-α secretion by monocyte/macrophages may be one of the mechanisms by which IL-10 inhibits IFNγ production (Wherry, 1991). However, TNF-α alone is not able to induce IFNγ production, and other cells besides monocytes and macrophages, including T and NK cells, also produce TNF-α, making it unlikely that inhibition of TNF-α production by IL-10 could be fully responsible for the effect on IFNγ production (Wherry, 1991). Indeed, interferons modulate the activity of virtually every component of the immune system and consequently enhance the body's ability to quell attacks by most disease-causing agents particularly viruses in the case of IFNγ (Johnson, 1994).

IL-10 AND EPSTEIN BARR VIRUS

The human IL-10 and murine IL-10 genes exhibit extensive sequence homology to an open reading frame in the Epstein Barr virus (EBV) genome, BCRF-1 (Vieira, 1991; Moore, 1990). Both IL-10 and the protein product of the BamHI fragment C rightward reading frame no.1 (BCRF-1) in the EBV genome, designated viral-IL-10 (vIL-10), have cytokine synthesis inhibitory activity on antigen- specific mouse T cell clones and on human peripheral blood lymphocytes (PBLs) activated by antigen and macrophages or by PHA and anti-CD3 monoclonal antibodies, respectively (Vieira, 1991; Hsu, 1990).

Both hIL-10 and vIL-10 show CSIF activity on mouse and human cells but vIL-10 has little of the mast-cell stimulatory activity of IL-10. This suggests that BCRF-1 may

encode only a subset of IL-10 activities (Thompson-Snipes, 1991). BCRF-1 is expressed late in the viral lytic cycle (Hudson, 1985). Although vIL-10 does not show all the properties described for cellular IL-10, it has retained the capacity to block cytokine synthesis, a function that could give a survival advantage to EBV (Fiorentino, 1989; Vieira 1991; Hsu, 1990; de Waal Malefyt, 1992).

Human and viral IL-10 each stimulate DNA replication in B lymphocytes activated via either their antigen receptor or their CD40 antigen (Banchereau, 1991; Rousset, 1991). IL-4 and IL-10 exhibit additive effects and induce a strong increase in the number of viable cells (Rousset, 1992). In addition, IL-10 induces activated B cells to secrete large amounts of IgG, IgA, and IgM, and the combination of IL-10 and IL-4 results in the secretion of the four immunoglobulin isotypes. Therefore, IL-10 may play an important role in the amplification of humoral responses (Mosmann, 1986; Mosmann, 1989; Fernandez-Botran, 1988; Rosen, 1977). It has been shown that IL-10 and vIL-10 each strongly block the antigen-specific proliferative responses of both T cells and $CD4^+$ Th1- or Th2-like T cell clones. The inhibitory effects on antigen-specific T cell proliferation were only observed when monocytes, but not EBV-LCL, were used as APC. In addition, it is observed that IL-10 and vIL-10 have strong downregulatory effects on both constitutive and IL-4 or IFNγ-induced class II MHC expression on monocytes, indicating that the strongly reduced proliferative T cell responses towards antigens are due to inhibition of the antigen-presenting capacity of monocytes through downregulation of class II MHC antigens on these cells (de Waal Malefyt, 1991).

IL-10 INTERACTION WITH IFNγ AND IL-12

The natural killer cell stimulatory factor or interleukin-12 (NKSF/IL-12) is a heterodimeric cytokine produced by monocytes/macrophages, B cells, and possibly other accessory cell types (Kobayashi, 1989; Wolf, 1991; Gubler, 1991; D'Andrea, 1992). IL-12 has effects on T and NK cells and enhances cytokine production and proliferation during cytotoxic activity (Chan, 1991; Perussia, 1992; Gately, 1991; Chehimi, 1992; Gately, 1992; Desai, 1992). IL-12 effectively enhances IFNγ production by T and NK cells. This effect is synergised with other IFNγ-inducers such as IL-2 and phorbol diesters for both T and NK cells. Particularly after exposure of these cells to antigens, alloantigens, mitogens, and anti-CD3 antibodies, target cells, immune complexes, and anti-CD16 antibodies for NK cells (Chan, 1992). Neutralizing anti-NKSF/IL-12 antibodies inhibit IFNγ production from human PBL induced by several stimuli. This could indicate that IL-12 is required for optimal IFNγ production by resting human lymphocytes (D'Andrea, 1992).

IL-12 also induces production of other cytokines by T and NK cells, including TNF-α (Perussia, 1992). IL-12 induces Th type 1 responses and inhibits the differentiation of IL-4 producing Th2 cells in the response of human PBLs to both allergens and bacterial antigens (Manetti, 1993). Anti-IL-12 antibodies have been shown to prevent Th1 differentiation (Hsieh, 1993), which suggests that IL-12 is a necessary factor for Th1 cell differentiation (D'Andrea, 1993).

IL-10 efficiently inhibits IL-12 production by human PBLs, IFNγ production by preventing the production of the IFNγ-inducing cytokine NKSF/IL-12 as well as the

costimulatory cytokine IL-1β, and TNF-α by accessory cells (Chan, 1991; Chan, 1992). In addition, IL-10 can act directly on human T cells, triggering a signalling pathway that specifically inhibits IL-2 synthesis (de Waal Malefyt, 1993).

IL-10 IN AIDS

The immune defects in AIDS patients are associated with a decrease in the number of $CD4^+$ T lymphocytes. The loss of $CD4^+$ T cells may be the result of infection with HIV (Lane, 1983; Fahey, 1984; Lane, 1985; Bowen 1985; de Wolf, 1988). However, before a severe depletion of $CD4^+$ cells occurs, a long asymptomatic period between infection and development of AIDS may be observed (Mathur-Wagh, 1985; Goedert 1986; Melbye, 1986; Taylor, 1986; El-Sadr, 1987). During this period, near normal numbers of $CD4^+$ cells are observed along with the absence of symptoms, positive delayed skin reactions, and positive *in vitro* proliferative responses to antigenic and mitogenic stimuli. Such a period may be followed by a progressive and sometimes rapid deterioration resulting in symptomatic AIDS (Clerici, 1989).

The hallmarks of HIV infection are immunosuppression and the onset of opportunistic infections. HIV induces dysfunction and eventual depletion of $CD4^+$ T cells. Interaction of HIV with $CD4^+$ cells involves an array of complex mechanisms which include the direct cytopathic effects of HIV on $CD4^+$ T cells, disruption of critical interaction between MHC proteins and molecules, defective signal transduction in $CD4^+$ T cells, interference with the normal cytokine network, and induction of autoimmune phenomena that may result in $CD4^+$ T cell destruction (Barre-Sinoussi, 1983; Gallo, 1984;

Amadori, 1990; Rosenberg, 1990; Clerici, 1993; Molina, 1990; Clerici, 1989; Viscidi, 1989; Clerici, 1991; Miedema, 1990; Habeshaw, 1992; Banada, 1992; Ameisen, 1991; Meyaard, 1992). It is not certain what contribution each of these mechanisms has in the defeat of the immune system seen in AIDS, but the effects of different pathogenic mechanisms probably vary considerably during each stage of infection. The demonstration of high levels of HIV in lymph node cells (LNC) and the low viral load in PBLs during clinical latency of HIV infection (Rosenberg, 1990; Miedema, 1990; Clark, 1991; Daar, 1991; Pantaleo, 1993b; Tenner-Racz, 1986; Pantaleo, 1993a; Embretson, 1993; Milovits, 1992; Graziosi, 1993), suggest that viral replication in LNCs and direct T cell killing by HIV play a significant role in the pathogenesis of AIDS (Klatzmann, 1984; Hirsch, 1992; Stanley, 1993). However the mechanism of $CD4^+$ T cell depletion in AIDS remains elusive. There is evidence that HIV infection causes $CD4^+$ T cell depletion by indirect mechanisms, such as the inhibition of immunoregulatory cytokines and apoptosis (Weiss, 1993; Gougeon, 1993; Banada, 1990), which may be more important than direct T cell killing.

T HELPER RESPONSES IN HIV INFECTION

Classification of cytokines into Th1 and Th2 types has helped in the elucidation of the mechanisms of resistance or susceptibility to infections (Sher, 1992). Preferential induction of cell-mediated immunity by intracellular pathogens including bacterial, viral, and protozoan infections (Scott, 1991; D'Andrea, 1992; Scott, 1994; Gazzinelli, 1993) and humoral immune responses by extracellular pathogens (Scott, 1991; Silvia, 1992; Heinzel,

1991; Heinzel, 1989; Mosmann, 1991) may be dependent upon the selective induction of Th1 or Th2 cell subsets respectively. Th1 cells produce IL-2 and IFNγ and induce cell-mediated immunity. Th2 cells produce IL-4, IL-5, and IL-10 and induce humoral responses (Mosmann, 1991; de Waal Malefyt, 1991 b; de Waal Malefyt,1991 a; Rousset, 1992; Howard, 1992; Burdin, 1993; Emilie, 1992; Benjamin, 1992; Fiorentino, 1989). These "class responses" are reciprocally controlled; Th1 cells inhibit Th2 cells and vice versa (Fiorentino, 1989). It has recently become clear that immunoregulatory cytokines IL-4, IL-10, and IL-12 induce differentiation of naive T helper cells (Th0) into Th1 and Th2 cells (Trinchieri, 1993; Hsieh, 1993; Scott, 1993).

Immunosuppression may also result from the release of immunoregulatory cytokines which influence the development of immune effector cells. The type of Th class generated in response to HIV antigens is not well characterized, alterations in the crossregulation of Th1 and Th2 class response may play a critical role in HIV immunopathogenesis (Miedema, 1988; Clerici, 1993). It may depend on the stage of HIV infection, degree of immunodeficiency and coexisting infections. Several groups have demonstrated that Th function is affected before a decrease in $CD4^+$ T cell count occurs (Clerici, 1989; Miedema, 1988; Shearer, 1992).

In summary, human IL-10 (hIL-10) is a pleiotropic cytokine exerting immunostimulatory and immunosuppressive effects. *In vitro* studies have shown that hIL-10 inhibits monocyte/macrophage/T- cell-dependent cytokine production, as well as antigen-specific proliferation of cultured T cell clones, while it enhances activated B cell

proliferation and antibody production. In addition, hIL-10 is produced by both Th1 and Th2 helper subsets, EBV-transformed B cells, macrophages, and monocytes. Inhibition of antigen-specific proliferation of human T cells by hIL-10 is mediated by downregulating the antigen-presenting ability of monocytes, which is associated with its downregulatory effects of the expression of class II MHC antigen on monocytes (Yssel, 1992). Hence, inhibition of both Th1-type cytokines and inflammatory cytokines seem to be the important biological properties of IL-10. IL-10 also exerts inhibitory effects *in vivo* as anti-mIL-10-treated mice exhibit elevation of T cell derived cytokines (Ishida, 1992).

OBJECTIVES

The present studies were undertaken to: (1) clone and express IL-10 (2) establish a biological and neutralization assay for IL-10 (3) examine the quantity of RNA of IL-10 and IFNγ present in the lymphocytes of HIV sero-poitive and HIV sero-negative individuals and (4) examine the quantity of IFNγ present in lectin stimulated prepheral blood lymphocytes of HIV sero-positive and HIV sero-negative individuals. To accomplish this objectives, recombinant hIL-10 and vIL-10 were produced in large quantities by transfecting either COS7 monkey kidney cells or *Spodoptera frugiperda* (SF9) cells to obtain IL-10 protein which was not commercially available at the time. The recombinant hIL-10 and vIL-10 were utilized to develop IL-10 biological and neutralization assays. This was followed by studying the effect of hIL-10 on IFNγ production by PBLs from HIV infected and normal individuals. Furthermore, expression

of both hIL-10 and IFNγ in PBLs of HIV infected and normal individuals were semi-quantitatively evaluated by reverse-transcriptase polymerase chain reactions and ELISA.

CHAPTER 2

Materials & Methods

Cell Lines, Culture Medium, and Plasmids

COS7 (SV 40 transformed African green monkey kidney cells) and B95-8 (EBV transformed marmoset leukocytes) cells were obtained from The American Type Culture Collection (ATCC, Rockville, MD) and cultured in complete Iscove's Dulbecco Modified Eagle's Medium (IDMEM)(Sigma, St. Louis, MO). The IDMEM medium used contained 10% fetal calf serum (P. A. Biological CO., Sydney, Australia), 100 IU/ml penicillin G (Ayerst, Don Mills, Ontario, Canada), and 50 μg/ml gentamicin (Schering, Pointe Clair, Quebec, Canada). The cells were incubated at 37°C with 5% CO_2 and the medium was changed every 3-4 days as required. SF9 insect cells were kindly provided by Dr. C. Y. Kang, University of Ottawa and cultured in EX-cell 400 medium (JRH-Bioscience, Lenexa, KS). The cells were incubated at 27°C with moisture and the medium was changed every 3-4 days as required. pcDSR-α 296 is a wild type mammalian expression vector of about 2,500 bp (Takebe, 1988). pcDSR-α-hIL-10 containing 537 bp of human

IL-10 cDNA and pcDSR-α-vIL-10 containing 510 bp of viral IL-10 cDNA were kindly provided by Dr. K. Moore of DNAX Institute (Moore 1990).

Transfection of COS7 Cells and Expression of IL-10 in the Mammalian System.

COS7 cells were transfected with pcDSR-α-hIL-10, pcDSR-α-vIL-10, and pcDSR-α 296 using DEAE-Dextran transient transfection method (Pharmacia, Uppsala, Sewden). COS7 cells were seeded and cultured on 100 mm tissue culture petri dishes (NUNC, Roskilde, Denmark) for 24 hours to reach 50% confluency. The recombinant plasmids (0.5 μg of DNA mixed with 100 μl of Tris-HCl (Sigma), pH 7.5) was mixed with an equal volume of DEAE-Dextran solution to the final volume of 100 μl. The DEAE-Dextran solution (2 mg/ml supplied in 0.9% NaCl) was used in three different concentrations : 0.5 mg/ml, 1.0 mg/ml, and 2.0 mg/ml. Medium was removed from the cells and the cells were then washed twice with isotonic Tris-HCl, pH 7.5. The DNA-DEAE-Dextran mixture was added to the cells drop-wise at the center of the plate and then incubated for 15 minutes at room temperature. The solution was carefully removed from the cells followed by a wash with isotonic Tris-HCl buffer, pH 7.5. Complete medium was added and the cells were incubated under normal growth conditions, using 5 μl of Dimethyl Sulfoxide, DMSO, (Sigma) per 3 ml of medium. The transfected cells were incubated for 72 hours. The supernatants of these cells were collected and saved at -70°C for subsequent use.

Cloning and Expression of hIL-10 and vIL-10 in the Baculovirus Expression System.

The B95-8 cells (infected with EBV) have been shown to express viral IL-10 (Hudson, 1985). These cells were employed to amplify viral IL-10 cDNA by PCR using specific primers for viral IL-10. The amplified PCR fragments were cloned in a Baculovirus expression vector, pACYM1, and were later expressed in SF9 insect cells.

B95-8 cells at 50% confluency were pipetted up and down in order to break cell clumps. 50 µl of the suspension was diluted in 450 µl of trypan blue dye (Sigma) for counting purposes. The cells were counted using a Hausser Scientific haemocytometer. Following calculation of cell numbers, DNA was extracted from 4×10^7 cells. Cells were harvested by centrifugation at 4000 rpm at room temperature for ten minutes and the pellet was washed twice with Dulbecoo's Phosphate Buffered Saline (PBS) (Sigma). After washing, the cells were incubated at 100°C for 10 minutes to disrupt cells and nuclear membranes. Proteinase K (Sigma) was added to the cells (to a final concentration of 0.12 mg/ml), and the extract was incubated at 50°C for 30 minutes. Proteinase K digestion was followed by incubation at 100°C for 10 minutes to denature proteinase K and DNA was precipitated by adding 0.1 volume of 3 M sodium acetate pH 5.2 and two volumes of absolute ethanol at -70°C for 30 minutes. DNA was recovered by centrifugation at 12000 rpm for 10 minutes. The DNA pellet was washed three times with 70% ethanol and DNA was dissolved in water to a final concentration of 1 µg/ul.

Viral IL-10, 537 bp long, was amplified by PCR (Perkin Elmer Cetus, Norwalk, CT) by employing the following primers: sense, 5'- ACA GGA TCC TAT AAA TAT GGA GCG AAG GTT AGT GGT -3';and antisense, 5'- TAG GAT CCT CAC CTG GCT

TTA ATT GTC ATG TAT GC -3' (Vieira, 1991). BamHI recognition sites were present upstream and downstream of vIL-10 during the synthesis of the oligonucleotides. The DNA was amplified under following conditions: 2.5 units of Amplitaq DNA polymerase, 150 pM of each of the above primers, 1 mM dNTP and 4 mM $MgCl_2$ in a total volume of 100 µl. The cycles used were: 1 cycle - 95°C, 5 minutes; 35 cycles - 95°C, 30 seconds, 60°C, 1.5 minutes; 1 cycle - 60°C, 7 minutes.

The PCR product amplified for vIL-10 was isolated by electrophoresis on 0.8% low melting point agarose gels, and purified by using GeneClean II (BIO/CAN Scientific INC., Sunnyvale, CA). The DNA fragment for vIL-10 was isolated and purified for cloning experiments.

The Baculovirus vector, pAcYM1 (9.5 kbp) (Kelly, 1982), was linearized by BamHI digestion. The linearized plasmid was purified on a 0.8% low melting point agarose gel. pAcYM1 and the vIL-10 amplimer were ligated using 5 units of ligase (Pharmacia) at room temperature overnight. All gel manipulations requiring visualization including isolation of the desired DNA fragment were carried out under long wave ultra-violet (UV) light to reduce the damaging effect of UV light.

Transformation:

a) Preparation of competent cells.

A Luria-Bertani (LB) agar (Sigma) plate was streaked with *E. coli* RR1 (kindly provided by Dr. C.Y. Kang) and incubated overnight at 37°C. A single colony was aseptically transferred into 200 ml of LB broth and was grown overnight at 37°C with

vigorous shaking at 300 rpm. The bacterial culture was cooled down to 4°C for 10 minutes and centrifuged at 4000 rpm for 10 minutes. The cell pellet was resuspended with ice-cold 0.1 M $CaCl_2$ and stored at 4°C for 5 minutes. The cells were centrifuged again and the pellet was resuspended in 8 ml of 0.1 M $CaCl_2$. An aliquot of cells was kept at 4°C to be used the following day whereas the remaining cells were frozen at -70°C with 15% glycerol.

b) Transformation

Fifty ng of ligated DNA (recombinant pACYM1-vIL-10) was added to 200 ul of competent RR1 cells and the sample was incubated at 4°C for 30 minutes, 42°C for 90 seconds, and finally 4°C for 2 minutes. The final volume of cells was brought up to 1 ml with LB broth and incubated at 37°C for 45 minutes. This latter process allowed the bacteria to recover and to express the antibiotic resistance gene (ampicillin resistance in this case) of the plasmid. The transformed cells were transferred onto LB agar plates containing 50 µg/µl ampicillin and incubated at 37°C overnight. A single colony was inoculated into 3 ml of LB containing 50 µg/µl ampicillin. The bacterial culture was vigorously shaken at 300 rpm 37°C overnight for small-scale preparation of plasmid DNA.

c) Small-Scale Preparation of Plasmid DNA.

The bacterial culture (1.5 ml) was centrifuged at 12,000 rpm for 30 seconds. The bacterial pellet was resuspended in 100 µl of Solution I containing 50 mM glucose, 25

mM Tris-HCl (pH 8.0),and 10 mM EDTA (pH 8.0) by vigorous vortexing. The bacterial cells were lysed at 4°C with 200 µl of NaOH (final concentrations of 0.2 N) and SDS (final concentration of 1%) followed by 150 µl of Solution III (3M potassium acetate, 11.5 ml glacial acetic acid). The lysate was centrifuged at 12,000 rpm for 5 minutes. An equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) was added followed by centrifugation at 12,000 rpm for 10 minutes. The aqueous phase was treated with two volumes of absolute ethanol at room temperature and centrifuged at 12,000 rpm for 5 minutes to precipitate DNA. The DNA pellet was washed two times with 70% ethanol, air-dried, and resuspended in 50 µl of water and stored at -20 °C.

d) Analysis of recombinant plasmid

The recombinant pAcYM1 plasmid was analyzed for correct orientation by restriction enzyme digestion using 1 U of each: BamHI, HinfI, EcoRI, and EcoRV per µg of DNA. The correct orientation is considered to be the 5'upstream of vIL-10 insert being ligated to the BamH1 site which is proceeded by ATG start site in the vector.

Restriction analysis and agarose gel electrophoresis indicated that vIL-10 cDNA in pAcYM1 was in the correct orientation. To confirm these observations, the recombinant vIL-10-pAcYM1 was kindly sequenced by Catherine Tsilfidis, Molecular Genetics, Children's Hospital of Eastern Ontario, using an automated DNA sequencer (Applied Biosystems, 373-A DNA Sequencer) with Sequenase version 2.0 (United States Biochemicals Corporation, Cleveland, Ohio) according to manufacturer's specifications

and using the following upstream and downstream primers : 5' - GTT GCT GAT ATC ATG GAG AT - 3' and 5' - TAC GTA CAA CAA TTG TCT GT - 3'.

e) Large-Scale preparation of plasmid DNA.

Large-scale preparation of plasmid DNA was performed using the QIAGEN (maxi-prep, Chatsworth, CA) plasmid preparation kit according to manufacturer's specifications. Purified DNA was redissolved in 100 µl of water.

f) Cotransfection of Baculovirus DNA and recombinant plasmid into SF9 cells.

Spodoptera frugiperda (SF9) cells (1.5 -2.0 x 10^6) were seeded in 35 mm petri dishes with 2.5 ml serum free EX-cell 400 medium. The cells were allowed to attach to the dish at room temperature for one hour. The serum free medium was then replaced with 1 ml of fresh complete EX-cell 400 medium. Six reaction mixtures containing Hepes buffer, different concentrations of recombinant plasmid DNA, Baculovirus DNA, and culture medium were prepared for cotransfection of SF9 cells (Table 1). Reaction mixes 1, 2, and 3 were designed to establish optimal conditions for transfection of recombinant plasmid DNA. Reaction mix 4 was used as a positive control for the transfection procedure, using wild type Baculovirus DNA and no recombinant plasmid DNA. Reaction mixes 5 and 6 were negative controls for buffer and cells respectively. The cotransfection buffer consisted of: 40 mM of 1M Hepes, 2 mM of 0.1 M Na_2HPO_4, 10 mM of 1M KCl, 280 mM of 4 M NaCl, in a total volume of 100 ml, pH 7.05.

Fifty μl of 2.5 M filter-sterilized $CaCl_2$ was added drop-wise to each reaction, except for the control cells. Each reaction was vortexed lightly and allowed to stand at room temperature for 30 minutes. The contents of the reaction tubes were then added drop-wise to the center of petri dishes containing SF9 cells. The petri dishes were transferred to a 27°C incubator for 3 hours. The reaction mixes were then removed and discarded. The cells were washed with 1.5 ml complete EX-cell 400 medium followed by the addition of EX-cell 400 medium to each petri dish. The petri dishes were incubated at 27°C for 4 days in a closed container with wet paper towels to keep the cell environment humid. The plates were checked daily by light microscope for the appearance of polyhedra as well as for enlarged rounded cells. The supernatants were collected from each plate after 4 days and centrifuged at low speed to separate the supernatant (containing virus) from the cells. A plaque assay was performed in duplicate to visualize possible Baculovirus recombinants (Summers, 1987). Thirty five mm petri dishes were seeded with 9×10^5 cells in 2.5 ml of serum-free EX-cell 400 medium, allowing cells to adhere for one hour. Medium was removed from the cells and 200 μl of serial dilutions of supernatants from reactions 3, 4, and 5 (Table 1) were added. Petri dishes were incubated at 27°C for one hour, with gentle rocking every 20 minutes. Equal volumes of 3% low melting point (LMP) agarose and complete medium were warmed to 40°C and mixed immediately prior to use yielding a final overlay of 1.5% agarose in complete medium. The supernatants containing Baculovirus were removed and 2 ml of

TABLE 1 - Composition of reaction mixtures used for cotransfection of Baculovirus DNA and recombinant pACYM1 (pACYM1-vIL-10) plasmid into SF9 cells

Reactions	1	2	3	4	5	6
Hepes Buffer[1]	570	570	570	570	570	570
Baculovirus DNA[2]	3	3	3	3	0	0
Plasmid[2]	1	2	4	0	0	0
Culture Medium[1]	950	950	950	950	950	x

1 - indicates micro-liters.
2 - indicates micro-grams.

overlay agar was added drop-wise to each 35 mm plate. The agarose was allowed to solidify for 20 minutes and 1 ml of complete medium was added to cover the overlay. Plates were incubated at 27°C for 3-6 days. During the incubation period the plates were visually screened for the appearance of recombinant plaques.

Assessment of biological activities of hIL-10 and vIL-10:

a) Proliferation Assays

PBLs (5×10^5 cells/ml) in IDMEM culture medium were distributed in a 96 well plate (Falcon, Lincoln Park, NJ) in a final volume of 200 µl per well. The supernatants containing recombinant hIL-10 or vIL-10 were diluted 1:10 and 1:100 and added to each well. Each well contained a total volume of 200 µl. Cells were incubated at 37°C for 30 minutes followed by the addition of either Phytohemagglutinin M-form (PHA-M) in a final dilution of 1:50 (Gibco/BRL, Gaithersburg, MD) or anti-CD3 antibodies in a final dilution of 1:200 (OKT3 cell line, ATCC). The cells were incubated at 37°C for 3 days. One hundred µl of supernatant from each well was harvested for analysis of IFNγ by ELISA.

For measurement of cell proliferation, 1 uCi of ^{3}H-Thymidine (Amersham, Life Science, Arlington Heights, IL) in 100 µl of fresh IDMEM medium was added and cells were incubated for another 7 hours at 37°C. The cells were then harvested and ^{3}H-

Thymidine incorporation was measured using a β counter (Beckman). Each test was carried out in triplicate.

b) Neutralization assays

PBLs (5 x 10^5 cells/ml) in IDMEM culture medium were distributed in a 96 well plate in a final volume of 200 µl per well. The supernatants containing recombinant hIL-10 and vIL-10 were added to each well in a final dilution of 1:10 and 1:100. Cells were incubated for 30 minutes at 37°C. The cells were stimulated with PHA-M in a final dilution of 1:50, human IL-2 (10 U/ml; AIDS Reference Center), and/or anti-TGF-β antibodies (2 µg/ml; R & D System, Minneapolis, MN). Twenty µl of anti-IL-10 antibody in varying dilutions of 1:10, 1:20, 1:50, and 1:100 was mixed with 100 µl of recombinant human and viral IL-10 supernatants (1:10 and 1:100 dilutions) and incubated for 30 minutes at 37°C and was then added to the stimulated cells.

c) Immunoprecipitation

The SF9 cells (1 x 10^7) cotransfected with vIL-10 and Baculovirus were washed with PBS and incubated for one hour in methionine-free EX-Cell 400 medium. After incubation, the medium was removed and replaced with 1 ml of methionine-free medium containing ^{35}S-Methionine (60 µCi/ml)(Amersham, Life Science). The cells were incubated for 3 hours followed by a wash with PBS and a rinse with rinse buffer (20 mM

Tris, 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 9.0). The cells were lysed with 1.0 ml of lysis buffer (20 mM Tris, 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1% (v/v) NP-40, 10% (v/v) glycerol, 1% aprotinin) for 10-20 minutes at 4°C.

The lysate was subjected to centrifugation for 12 minutes at 12,000 rpm. Rabbit anti-IL-10 polyclonal antibody was added to the lysate at dilutions of 1/100 to 1/500 in a final volume of 200 µl. Reactions were incubated for a minimum of 30 minutes with rotation, at 4°C . Protein A-Sepharose 4B beads (100 µl) (Sigma) equilibrated in RIPA buffer, were added to the lysate and incubated for a further 30 minutes at 4°C while rotating. The beads were washed three times with wash buffer (100 mM Tris, 500 mM LiCl, 1% (v/v) 2-mercaptoethanol). After the last wash the beads were resuspended in wash buffer, boiled for 2 minutes, centrifuged and supernatants were electrophoresed in a 10% SDS-polyacrylamide gel.

Isolation of PBLs

Peripheral blood mononuclear cells were isolated from anti-coagulated blood samples obtained from 35 human immunodeficient virus sero-positive patients (HIV^+) and 15 HIV sero-negative individuals (HIV^-). Polymorphprep reagent (Nycomed, Oslo, Norway) was used for separation as follows. Six ml of blood was layered carefully over 5 ml of polymorphprep in a 15 ml centrifuge tube. The samples were centrifuged at 500 Xg for 30 minutes at 22°C in an IEC Centra-8R centrifuge. After centrifugation, two

leucocyte bands were visible. The top band consisted of mononuclear cells and the lower band consisted of polymorphonuclear cells. Erythrocytes were pelleted. The mononuclear cells were harvested and washed twice with PBS at 400 Xg for 10 minutes at 22°C. The cell pellet was frozen at -70°C for subsequent RNA extraction and reverse transcriptase-polymerase chain reaction.

RNA isolation

The RNA of all individuals was extracted using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to manufaturer's specifications. The total RNA pellet was then air dried for 10 minutes and resuspended in 20 µl of autoclaved distilled water and stored at -70°C for subsequent use.

Semi-quantitative Reverse Transcriptase-Polymerase chain reaction

The message for IL-10 and IFNγ were detected in the unstimulated PBLs from HIV infected and normal individuals by a very sensitive semi-quantitative RT-PCR. The RNA was first reverse transcribed to cDNA and then the cDNA was ampilified by polymerase chain reaction. Generation of cDNA was performed using 2.5 U/µl cloned Moloney murine leukaemia virus reverse transcriptase (Gibco/BRL), 2.5 mM $MgCl_2$, 1 mM each of the dNTP, 1 U/µl RNase inhibitor, and 2.5 µM random hexamers in a final volume of 20 µl. The reaction was carried out under the following conditions: 42°C for

15 minutes followed by 94°C for 5 minutes and 5°C for 5 minutes. Five µl of the cDNA was subsequently ampilified for each of IL-10, IFNγ, and β-actin using 2.5 units of AmpiliTaq DNA polymerase, 150 pM of each of the 5' and 3' primers, 1 mM nucleotides, and 4 mM $MgCl_2$ in a total volume of 25 µl. The oligonucleotide primer sequences for IL-10, IFNγ, and β-actin are listed as follows. IL-10: sense; 5'-AAG GAT CCA TGC ACA GCT CAC TGC-3'; antisense; 5'-CTG CTC CAC TGC CTT GCT CTT ATT-3'. IFNγ: sense; 5'-ATG AAA TAT ACA AGT TAT ATG TTG GCT TT-3'; antisense; 5'-GAT GCT CTT CGA CCT CGA AAC AGC AT-3'. β-actin: sense; 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'; antisense; 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'. Samples were heated for 5 minutes at 95°C. Ampilification was carried out with 35 cycles of 95°C for 30 seconds, 60°C for 1.5 minutes and a final extension was performed at 60°C for 7 minutes.

Detection of IL-10, IFNγ, and β-actin RT-PCR Products by Southern Transfer and Hybridization:

a) Electrophoresis

PCR products (15 µl) were electrophoresed in a 1% agarose (Gibco/BRL) gel containing 0.5 µg/ml ethidium bromide, and subjected to electrophoresis at 100 volts for

approximately 2 hours in a large LKB/Pharmacia electropherosis box and gels were photographed using Polaroid type 667 film.

b) Southern Transfer

Following electrophoresis, the DNA was denatured in 0.4 N NaOH for 30 minutes at room temperature. The DNA was then transferred overnight by capillary action to positively charged nylon membrane (ICN Biotrans, Costa Mesa, CA). The following day the nylon membrane was neutralized by submerging in 2X SSC and 0.2 M Tris (pH 7.5) for 15 minutes on a shaker. In order for the DNA to be permanently attached to the membrane, the membrane was baked at 80°C in an oven for 30 minutes.

c) Preparation of radioactive probe

Approximately 25 ng of DNA template was labelled using a Prime-It kit (Stratagene, La Jolla, CA) according to the manufacturer's specifications. DNA templates were 1) human IL-10 cDNA (HindIII/BglII fragment of pcDSR-α-IL-10), 2) IFNγ and β-actin PCR products ampilified by RT-PCR employing the oligonuclotide primers described above. To separate the labelled DNA from unincorporated radioactive nucleotides a NICK column (Pharmacia) was used according to the manufacturer's specifications. NICK Columns contain Sephadex G-50 DNA for separation of nick-translated DNA from unincorporated ^{32}P-labelled nucleotides. To assess the specific

activity of the DNA probe, 2 µl of each of the purified probes and the last columnwash were counted in a Beckman (LS 7000) scintillation counter.

d) Hybridization

The membranes were pre-hybridized for 3 hours at 65°C in 10 ml of 10% PEG hybridization solution (10% PEG, 1.5X SSPE, and 7.0% SDS). The pre-hybridization solution was discarded and 10 ml of fresh hybridization solution containing 5 x 10^6 counts/ml of the probe were added to the nylon membrane. Two hundred and fifty µg/ml of salmon sperm was added to the hybridization solution along with the probe to reduce the background during hybridization. Hybridization was carried out at 65°C for 18 hours. The nylon membrane was washed five times with 2X SSC, 0.1% SDS at room temperature for 10 minutes followed by three to seven washes with 0.1X SSC , 0.1% SDS at 65°C for 30 minutes each. The membranes were exposed to X-ray film (Kodak XAR-5) for 1- 72 hours.

Measurement of IFNγ by ELISA

To determine the ability of stimulated PBLs from HIV infected individuals to produce IFNγ the cells were cultured at a concentration of 2 x 10^6 cells/ml. The cells were stimulated with PHA-M in a final dilution of 1:50. The supernatants were harvested after 24, 48, and 72 hours and were frozen at -70°C. A sandwich ELISA was employed

to quantitate the IFNγ produced by PBLs. Two different monoclonal antibodies recognizing two distinct IFNγ epitopes were used. Monoclonal antibody γ3-11.1 (ATCC) at a concentration of 5 μg/ml in PBS was used for coating the plates (Nunc Immuno-mopdules) overnight at 4°C. The plates were blocked with 5% skimmed milk in PBS-Tween 20 (0.05%). IFNγ was detected by a second biotynalated monoclonal antibody B-24 (UBI-Olympus) at a concentration of 5 μg/ml in PBS-Tween 20. Streptavidin conjugated with horseradish peroxidase (Jackson Immuno Research, Mississauga, Ontario, Canada) was used to develop the colour reaction and absorbance was measured at 540 nm. Recombinant IFNγ obtained from BioSource International was used concurrently as a standards for quantitation. PBLs from HIV sero-negative individuals were used as negative controls. The sensitivity of this assay was 16 pg/ml.

CHAPTER 3

Results

PRODUCTION OF RECOMBINANT HUMAN AND VIRAL IL-10.

During the initial part of this study, IL-10 was not available commercially. Therefore, IL-10 was produced in the laboratory by transfecting IL-10 cDNA into COS7 and SF9 cells.

Transfection of hIL-10 and vIL-10 in COS7 cells

Recombinant human and viral IL-10 cDNA in the mammalian expression vector pcDSR-α (pcDSR-hIL-10 and pcDSR-vIL-10) were transfected into COS7 cells growing in their log phase (24 hours culture) at 50% confluency (Moore, 1990). The plasmid pcDSR-α-296 was used as a negative control for transfecting plasmids. The transfection conditions were optimized, using three different concentrations of DEAE-Dextran and three different concentrations of cells. DMSO was utilized to increase the permeability of the cell membrane and consequently the effectiveness of the transfection. The culture

supernatants of COS7 cells transfected with hIL-10 and with vIL-10 were analyzed for biological activities.

Construction of recombinant Baculovirus vector pACYM1-IL-10

B95-8 cells contain the viral IL-10 gene due to the presence of EBV. Therefore, these cells were used as a source of vIL-10. In order to clone vIL-10 sequences, DNA was isolated from B95-8 and was amplified using oligonucleotide primers specific for vIL-10 (as described in Materials & Methods). The vIL-10 DNA, with two BamHI recognition sites at each end, was inserted into the BamHI site of pACYM-1.

The vIL-10 : pACYM1 ligation product was then utilized to transform RR1 *E. coli*. Small-scale plasmid preparations were used to test the presence of the desired vIL-10 : pACYM1 recombinant plasmid DNA. A series of restriction enzymes were employed to determine the presence as well as the orientation of the vIL-10 insert in the Baculovirus expression vector pACYM-1. The digestion pattern was indicative of the presence of a 537 bp insert in the appropriate orientation.

Sequence analysis confirmed our preliminary observation. Therefore, the vIL-10 gene subcloned into pACYM1 was in the correct orientation and could be used for later manipulations in the Baculovirus expression system.

SF9 cells were transfected with the recombinant pACYM1-vIL-10 (Kelly 1982). After 4 days, the supernatants, which contain the Baculovirus, were harvested. The supernatant of transfected SF9 cells were examined for the presence of vIL-10 protein by a protein gel (Fig.3) and the biological activities by a proliferation assay (Fig.1).

ASSESSMENT OF IL-10 BIOLOGICAL ACTIVITY IN IL-10 TRANSFECTED COS7 and SF9 CELLS:

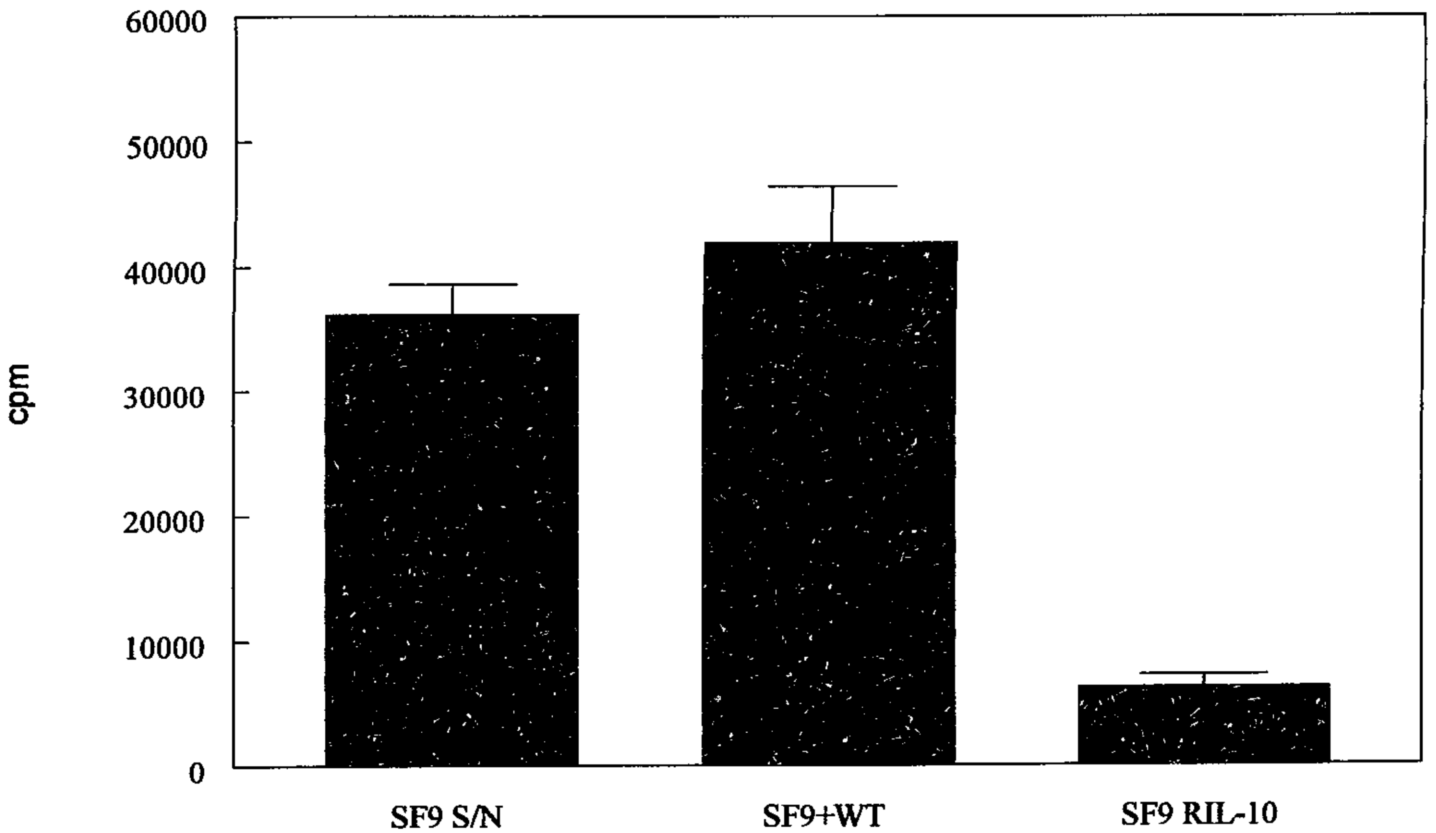
The biological activities of the supernatants of cultures from various transfectant cells were assayed by the following methods.

Inhibition of proliferation of PHA activated PBLs by recombinant viral IL-10.

PBLs obtained from a normal individual were stimulated with PHA in the presence and absence of various dilutions of supernatants of SF9 cells transfected with recombinant pACYM1-vIL-10, SF9 cells transfected with wild type plasmid pACYM1 (transfection negative control), and media (SF9 cells negative control). Cell proliferation was measured by ^{3}H-Thymidine incorporation. Supernatants from SF9 cells transfected with pACYM1-vIL-10 inhibited the proliferation of PHA stimulated PBLs in comparison to supernatants collected from SF9 cells either cultured alone or transfected with pAcYM1 plasmid (Fig.1).

Therefore, vIL-10-containing supernatants from SF9 cells inhibited proliferation of PHA stimulated PBLs, whereas supernatants of control SF9 cultures did not modulate PHA induced proliferation of PBLs. This suggests that the inhibition was specifically due to recombinant vIL-10. Such inhibition of proliferation was shown to be specific and dose dependent. Together these results suggest that vIL-10 transfected SF9 cells secreted vIL-10.

FIGURE 1 - Proliferation Assay for Recombinant vIL-10 in PHA Induced PBLs. Supernatants from SF9 cells transfected with wild type pACYM1 and recombinant pACYM1-vIL-10 were assayed for their inhibition of PBLs proliferation. SF9 s/n - denotes PBLs stimulated with supernatant fluid from non-transfected SF9 cells; SF9+w.t. - denotes PBLs stimulated with supernatant fluid from SF9 cells transfected with pAcYM1 plasmid; SF9 RIL-10 - denotes PBLs stimulated with supernatant fluid from SF9 cells transfected with pACYM1-vIL-10. CPM is counts per minute of radioactivity. The cap lines on the graphs represent error bar.

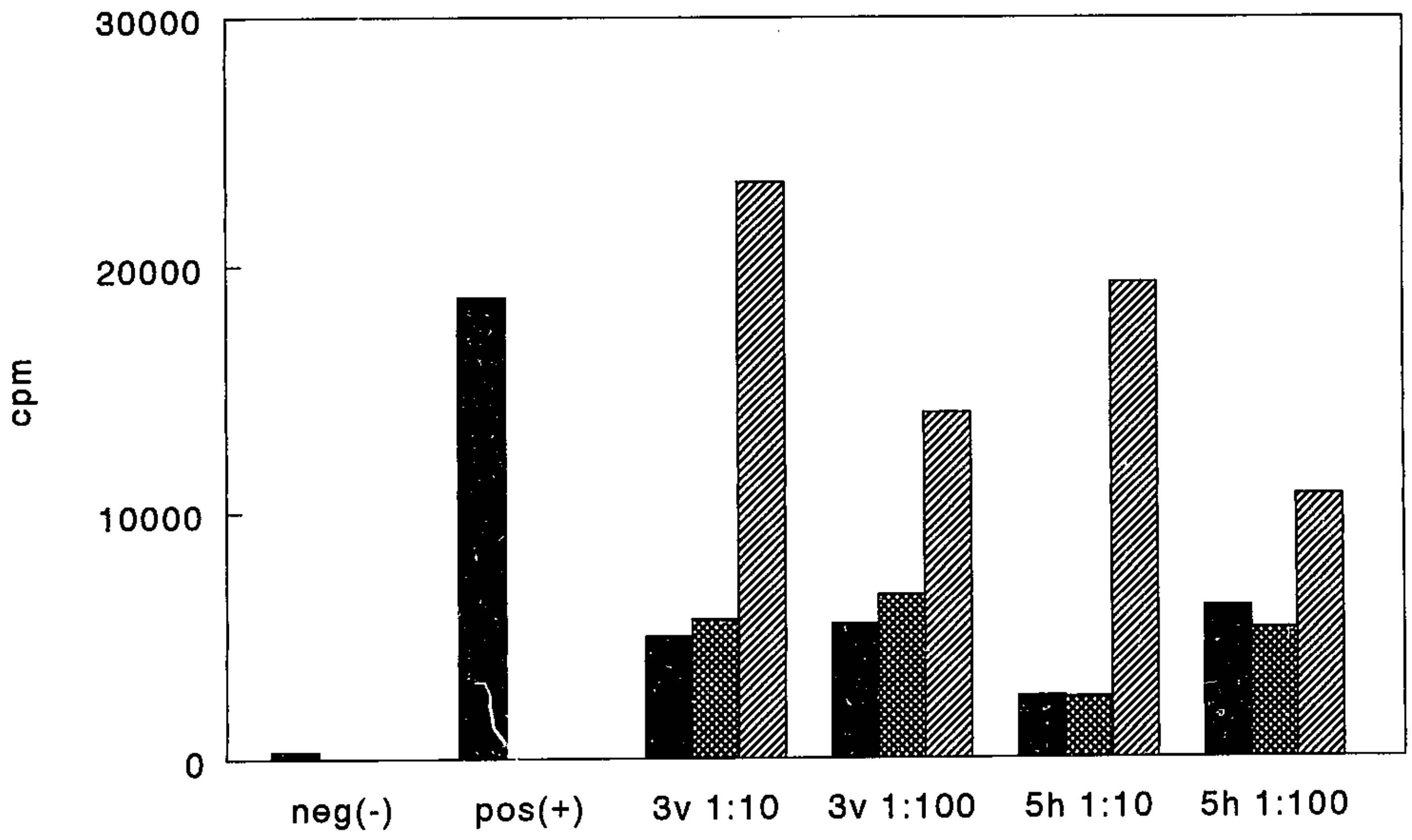

cpm
60000
50000
40000
30000
20000
10000
0
SF9 S/N
SF9+WT
SF9 RIL-10

Neutralization assays, neutralization of bioactivity in hIL-10 and vIL-10 transfected COS7 supernatants:

To determine whether the inhibition of PHA-stimulated PBLs by recombinant IL-10 was due to IL-10, anti IL-10 antibodies in different dilutions were mixed with recombinant IL-10 and were added to stimulated PBLs. These PBLs were first stimulated with PHA, IL-2, and anti-TGF-β antibody as described in the Materials and Methods. Anti-TGF-β antibodies were added to inhibit any TGF-β activity in the COS7 supernatants (Coligan, 1991). Also, IL-2 has been shown to increase IL-10 secretion (Coligan, 1991). Therefore, IL-2 was added to saturate the IL-2 receptors. After 24 hours of incubation at 37°C, cells were pulsed with ^{3}H-Thymidine and further incubated for another 16 hours. The results of these experiments are shown in Figure 2.

The maximum inhibitory activity of both supernatants containing recombinant human IL-10 (designated as 5h) and recombinant viral IL-10 (designated as 3v) was observed with 1:10 dilutions. IL-10-transfected COS7 cell supernatants inhibited PHA-M-induced proliferation of human PBLs as expected. Anti-IL-10 antibodies neutralized the IL-10 induced proliferation inhibition, in a dose dependent fashion. The anti-hIL-10 antibody neutralized the hIL-10 (5h) and resulted in a four fold (in the case of the 1:10 dilution of 5h) and a two fold (in the case of 1:100 dilution of 5h) increase in proliferation as compared to the control serum from the same rabbit. In the case of vIL-

FIGURE 2 - Neutralization of human and viral IL-10 obtained from COS7 transfections by polyclonal rabbit anti-hIL-10 and anti-vIL-10 antibodies. Neutralization assays were carried out as described in Materials & Methods. (3v) indicates recombinant vIL-10 and (5h) indicates recombinant human IL-10. The dilutions of the supernatants are indicated below each group of bars. The Mitogen of choice was PHA-M. The readings are ^{3}H- Thymidine incorporation counts per minute. The black bars indicate PBLs stimulation controls; the cross-hatched bars indicate PBLs treated with pre-immunized rabbit serum; the crossed bars indicate PBLs treated with post-immunized rabbit serum. CPM is counts per minutes of radioactivity. The cap lines on the graphs represent error bars.


30000
20000
10000
0
cpm
neg(-)
pos(+)
3v 1:10
3v 1:100
5h 1:10
5h 1:100

10 similar observations were made. The anti-vIL-10 antibody was able to neutralize vIL-10 (3v) and led to eight fold (in case of 1:10 dilution of 3v) and twofold (in case of 1:100 dilution of 3v) increases in proliferation. Neutralizations of IL-10 with the anti-IL-10 antibodies therefore, revealed the specific inhibitory effect of IL-10 on PBLs proliferation. Furthermore, these results suggest that the transfected COS7 cells were secreting viral IL-10. These results were further confirmed by a IFNγ ELISA described in Table 3.

Immunoprecipitation and Western blot analysis.

In order to further examine the presence of vIL-10 in SF9 insect cells cotransfected with pACYM1-vIL-10 and Baculovirus DNA an immunoprecipitation analysis was performed using rabbit polyclonal anti-vIL-10 antibodies. The immunoprecipitation showed the presence of two predominant proteins, at <20 kD and <14 kD, respectively, in transfected SF9 cells in comparison to SF9 cells transfected with pACYM1. Reported sizes of BCRF-1, vIL-10, proteins are 20, 19, and 17 kD (Moore 1990) (Fig.3).

EXPRESSION OF IL-10 IN HIV INFECTED INDIVIDUALS

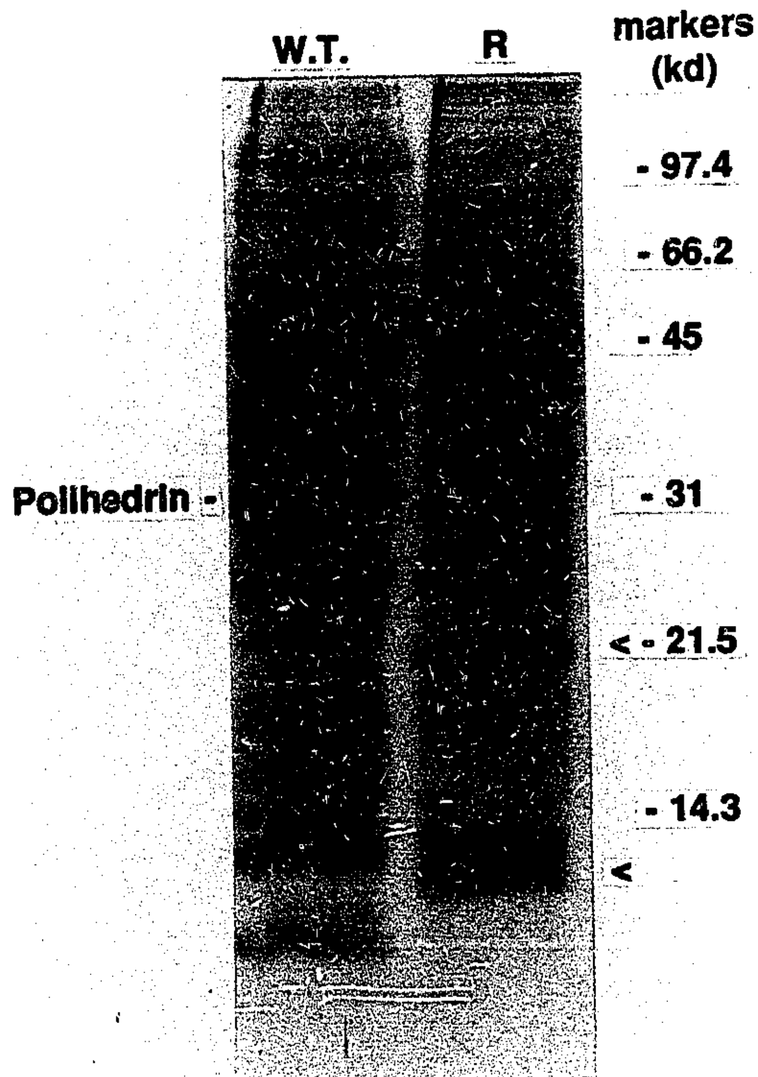
HIV^+ individuals exhibit dramatic loss of $CD4^+$ T cells during the course of disease progression. Furthermore, sequential loss of immune responses to recall antigens, allo-antigen, and mitogens are observed which have been attributed to the dysregulation of cytokines such as IL-2 and IL-4 produced by Th1 and Th2 cells respectively.

TABLE 2 - IFNγ ELISA readings of a biological assay of human and viral IL-10-transfected COS7 supernatants

	Pre-immune	Anti-hIL-10	Anti-vIL-10
vIL-10 (1:10)	0.075	0.015	0.26
vIL-10 (1:100)	0.026	0.003	0.21
hIL-10 (1:10)	0.009	0.20	0.13
hIL-10 (1:100)	0.008	0.26	0.05

PBLs were treated with; pre-immunized rabbit anti-serum, rabbit polyclonal anti-vIL-10 antibodies, or rabbit polyclonal anti-hIL-10 antibodies. This assay was repeated three times and the above values are the mean average of the repeats. Supernatants were collected and assayed for IFNγ production by a sandwich ELISA as described in Materials & Methods. Values indicate IFNγ levels in pg/ml.

FIGURE 3 - Protein gel of SF9 cells cotransfected with Baculovirus and pACYM1-vIL-10. SF9 insect cells were cotransfected with recombinant pACYM1-vIL-10 plasmid and Baculovirus DNA, were lysed and poly peptides were immunoprecipitated with rabbit polyclonal anti-vIL-10 antibodies. SF9 cells transfected with recombinant plasmid exhibit the presence of two different proteins at <20 and <14 kDa in comparison to SF9 cells transfected with pACYM1 plasmid alone which exhibit polyhedrin protein at 31 kDa.



W.T.
R
markers
(kd)
- 97.4
- 66.2
- 45
Polihedrin -
- 31
< - 21.5
- 14.3
<

It has been suggested that HIV^+ patients may exhibit predominance of Th2 type cytokines and loss of protective cell mediated immune responses. Accordingly IL-10, a Th2 type cytokine, seems to be upregulated in HIV infections. Hence, to understand the role of IL-10 in HIV infection, we measured IL-10 expression in unstimulated PBLs of HIV infected and normal individuals by means of semi-quantitative RT-PCR and ELISA. IL-10 is also shown to suppress the synthesis of IFNγ by Th1 cells in the PBLs of HIV infected individuals. In order to demonstrate IL-10 and IFNγ levels in HIV infection, standard RT-PCR procedures were optimized.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

PBLs from 35 HIV infected patients and fresh PBLs from 15 normal control individuals were obtained for RNA isolation. The RNA was reverse transcribed and IL-10, IFNγ, and β-actin sequences were amplified under optimized conditions as described in the Materials and Methods. RT-PCR was performed for β-actin, a housekeeping gene, for two reasons : first, to demonstrate the quality of the isolated RNA and secondly to provide a semi-quantitative measure of IL-10 and IFNγ expression for accuracy and reproducibility of RT-PCR analysis. β-actin RT-PCR was performed with known quantities of RNA using different numbers of PCR cycles. Following Southern blot analysis, autoradiographs were scanned by densitometry. The results are shown in Figures 4-7. Saturation for PCR amplifications and hybridization was observed at 35 cycles and at concentrations of total RNA exceeding 0.15 μg. Therefore, RT-PCR for β-actin expression was dependent on the amount of RNA and the number of

FIGURE 4 - β-actin RT-PCR is RNA dose dependent and PCR cycle dependent. Total RNA from in the range of 10-100 ng from PBLs of a normal individual was reverse transcribed. The generated cDNA was specifically amplified for β-actin by 20, 25, and 30 cycles of PCR under same conditions. PCR amplified products were analyzed by hybridization using probes specific for β-actin. Radioactive blots were exposed to X-ray films.

100 ng	50 ng	25 ng	10 ng	100 ng	50 ng	25 ng	10 ng	100 ng	50 ng	25 ng	10 ng

ß ACTIN →

20 Cycles	25 Cycles	30 Cycles

FIGURE 5 - β-actin RT-PCR is RNA dose dependent and PCR cycle dependent. Total RNA in the range of 25-200 ng from PBLs of a normal individual was reverse transcribed, PCR amplified (30 and 35 cycles), and analyzed as described in the legend to Figure 4. The relative amounts of amplified product were measured by densitometry. The black bars indicate 30 cycles of PCR and the cross-hatched bars indicate 35 cycles of PCR amplification. The mean values of three repeats of the experiments were used for this figure.

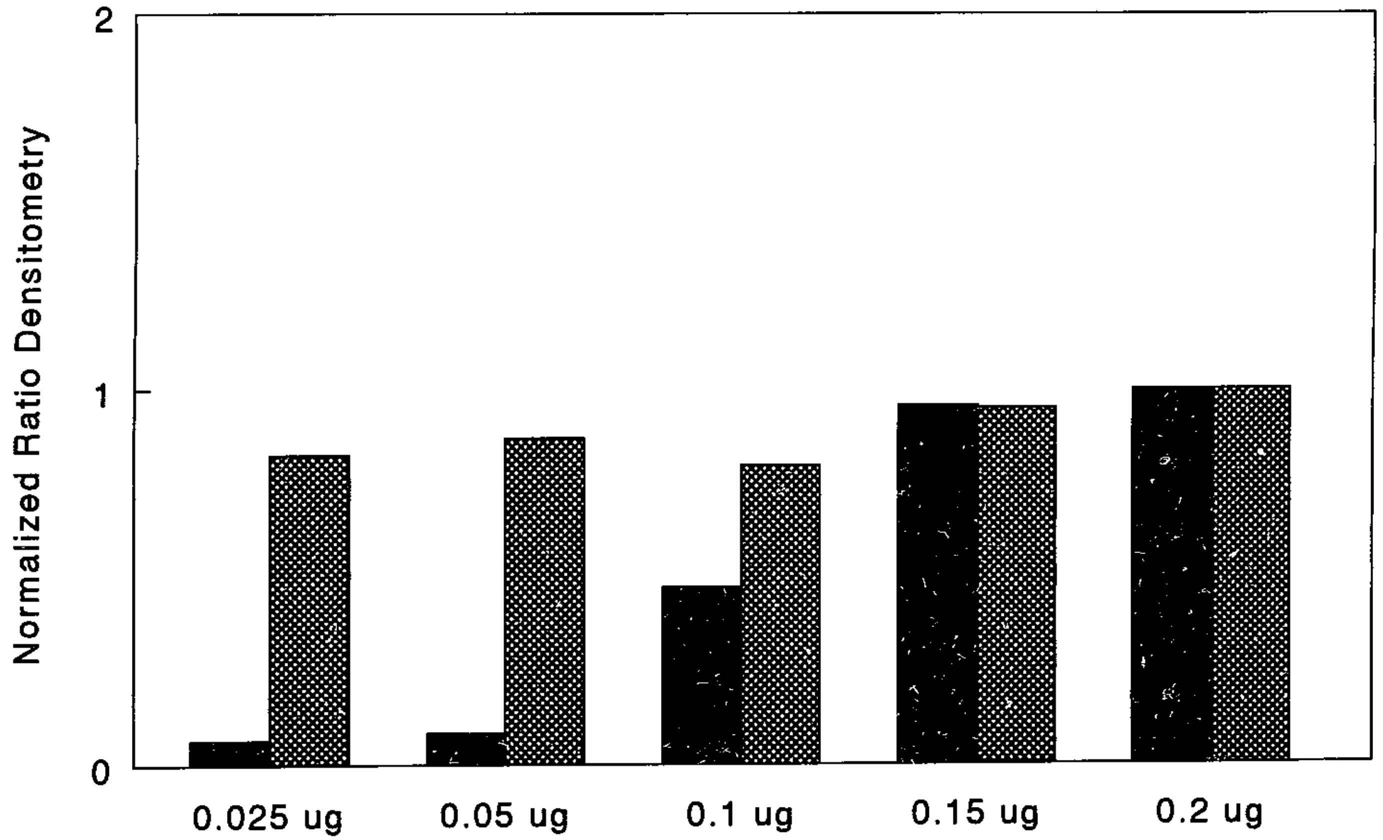

Normalized Ratio Densitometry
2
1
0
0.025 ug
0.05 ug
0.1 ug
0.15 ug
0.2 ug

FIGURE 6 - β-actin RT-PCR is RNA dose dependent and PCR cycle dependent. Total RNA in the range of 10-100 ng from PBLs of a normal individual was reverse transcribed, PCR amplified (25, 30, and 35 cycles), and was analyzed as described in the legends to Figures 4 and 5.

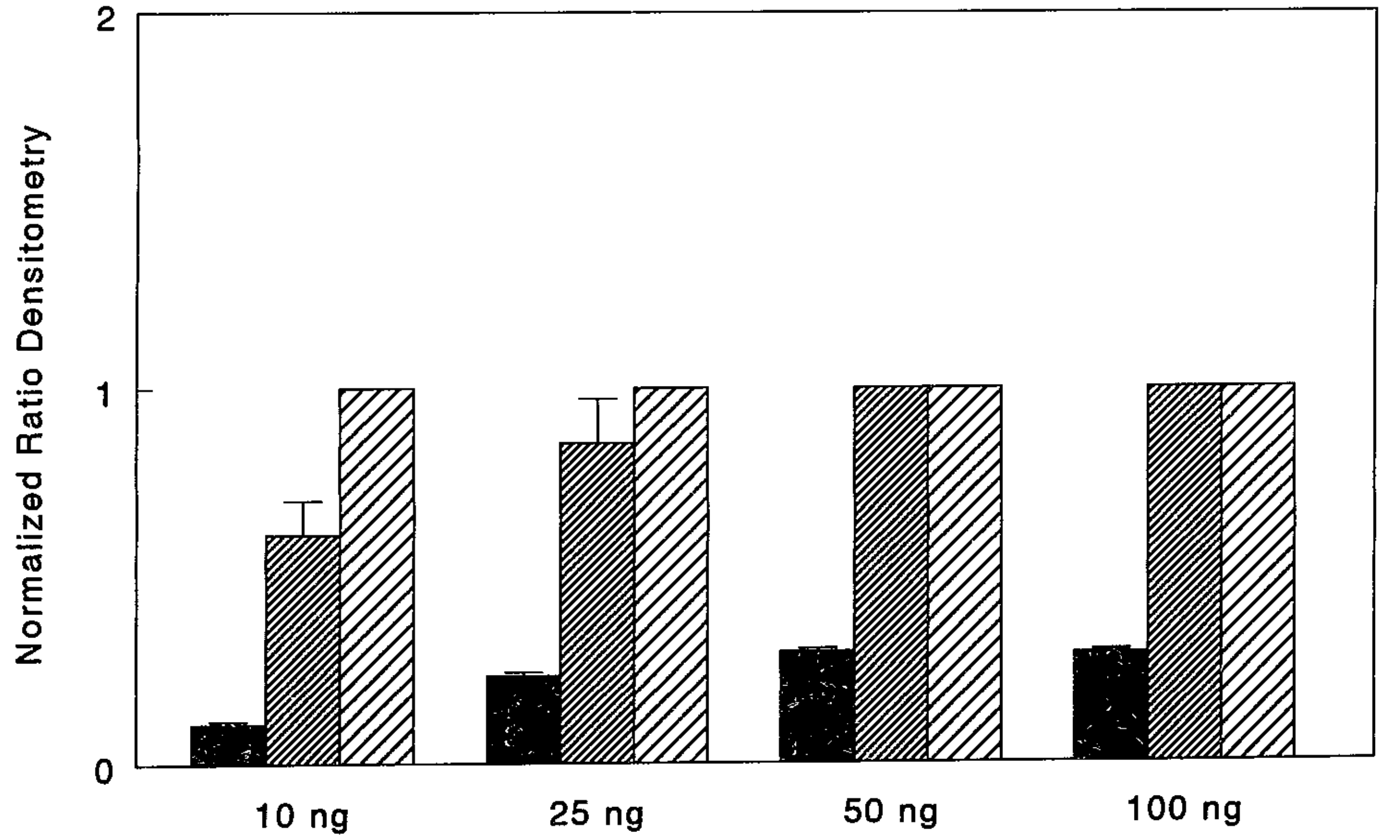
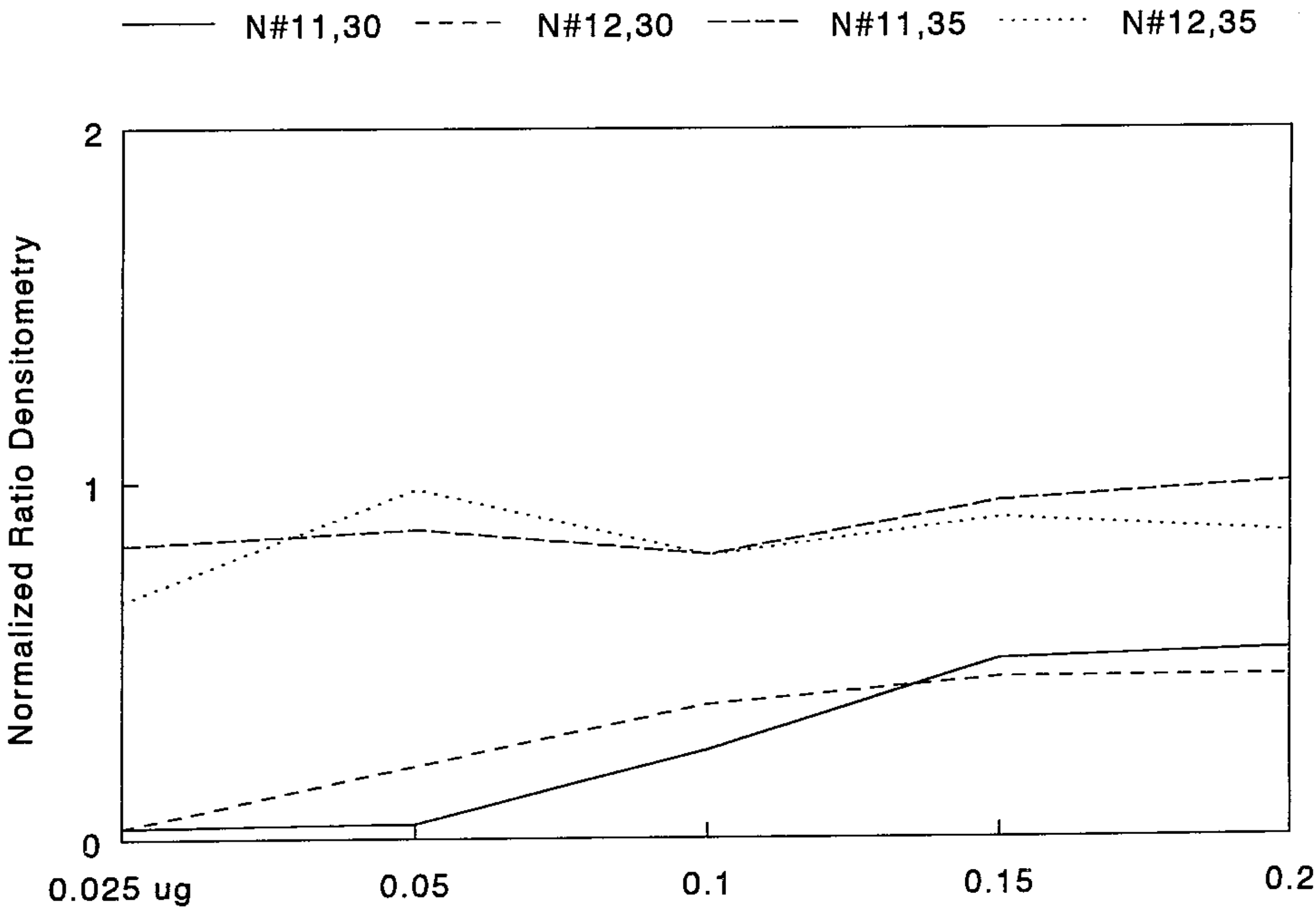

Normalized Ratio Densitometry
2
1
0
10 ng
25 ng
50 ng
100 ng

FIGURE 7 - β-actin RT-PCR is RNA dose dependent and PCR cycle dependent. Total RNA in the range of 25-200 ng from PBLs of two normal individuals (N#11 & N#12) were reverse transcribed, PCR amplified (30 and 35 cycles), and was analyzed as described in the legends to Figures 4 and 5.


N#11,30
N#12,30
N#11,35
N#12,35
Normalized Ratio Densitometry
2
1
0
0.025 ug
0.05
0.1
0.15
0.2

amplifying cycles used for PCR. Thus, in further experiments, 30 amplifying cycles for β-actin PCR were employed.RT-PCR for IL-10, IFNγ, and β-actin using RNA samples of HIV sero-positive and control normals was performed (Fig.8). A ratio of IL-10 PCR product to PCR product of β-actin expressed in densitometry units, was determined by Image-Pro Plus software (Fig.9). This analysis revealed significantly higher levels of IL-10 message in HIV sero-positive patients with more than 400 $CD4^+$ T cell counts (0.751 ± 0.623 densitometry units) and less than 400 CD4+ T cell counts (1.19 ± 0.885 densitometry units) than in normal controls (0.051 ± 0.073 densitometry units, $p<0.001$) (Fig.9). Nine out of ten HIV-infected patients exhibited approximately fifty-fold higher IL-10 mRNA levels than did normal controls. In similar experiments, the ratio of IFNγ RNA to β-actin RNA from patients with less than 400 $CD4^+$ counts (0.0312 ± 0.014 densitometry units) was significantly lower than in normal controls (0.614 ± 0.374 densitometry units, $p<0.001$) (Fig.10). Furthermore, there was forty to fifty fold less IFNγ cDNA amplified from unstimulated PBLs from HIV sero-positive patients with less than 400 $CD4^+$ T cells than for five normal controls . No significant differences were observed in the amounts of DNA amplified from IFNγ mRNA in PBLs in patients with more than 400 $CD4^+$ T cells (IFNγ/β-actin = 0.363 ± 0.153) than for normal controls (0.614 ± 0.374).

In order to confirm these observations, the experiments were repeated two more times with additional samples. In the second data set, PBLs from eleven HIV sero-positive patients and from six normal controls were tested using RT-PCR to estimate the levels of IL-10, IFNγ, and β-actin mRNA (Fig.11).

FIGURE 8 - Southern Transfer of IL-10, IFNγ, and β-actin RT-PCR of HIV Immunosuppressed and Normal Individuals. Total RNA from ten HIV sero-positive patients and five HIV sero-negative individuals were reverse transcribed. Equal amounts of cDNA from each individual were amplified using oligonucleotide primers for IL-10, IFNγ, and β-actin. PCR amplified products were electrophoresed on agarose gel and analyzed by hybridization using probes specific for IL-10, IFNγ, and β-actin. Radioactive blots were exposed to X-ray films. C1 to C5 denote HIV sero-negative individuals. P1 to P10 denote HIV sero-positive patients.

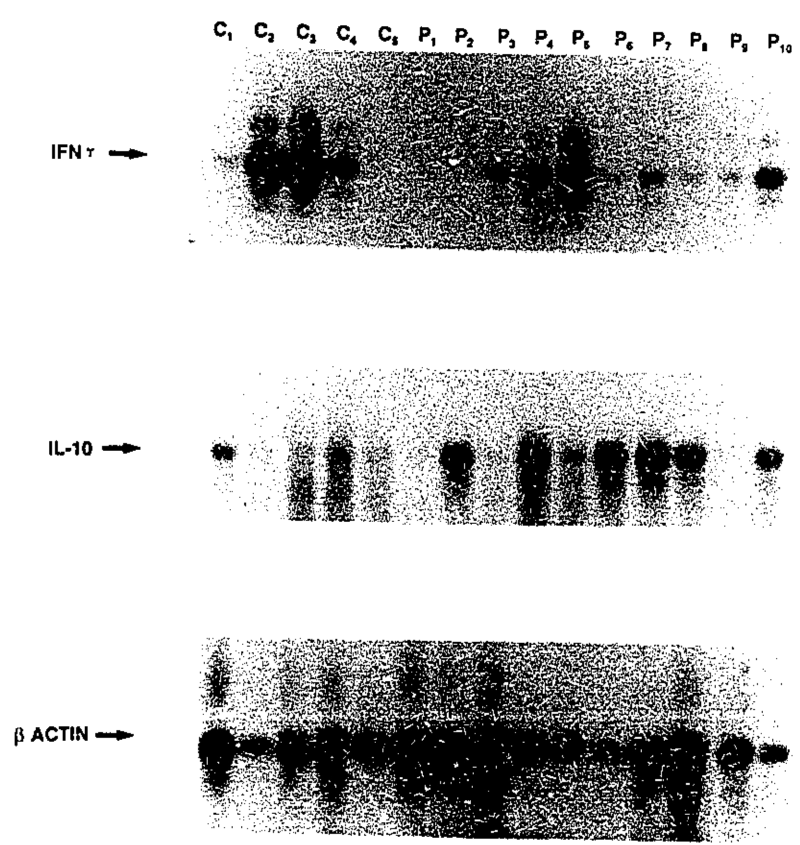

C1 C2 C3 C4 C5 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10
IFN γ
IL-10
β ACTIN

FIGURE 9 - IL-10 Semi-quantitative RT-PCR. IL-10 PCR amplified bands as demonstrated by autoradiography were analyzed by densitometry and assigned arbitrary units. The values representing the amount of amplified IL-10 cDNA was normalized with respect to values for amplified amounts of β-actin cDNA. The black bars represent the mean average of IL-10 normalized densitometry readings of individuals with various $CD4^+$ counts. The cap lines on the graphs represent error bars.

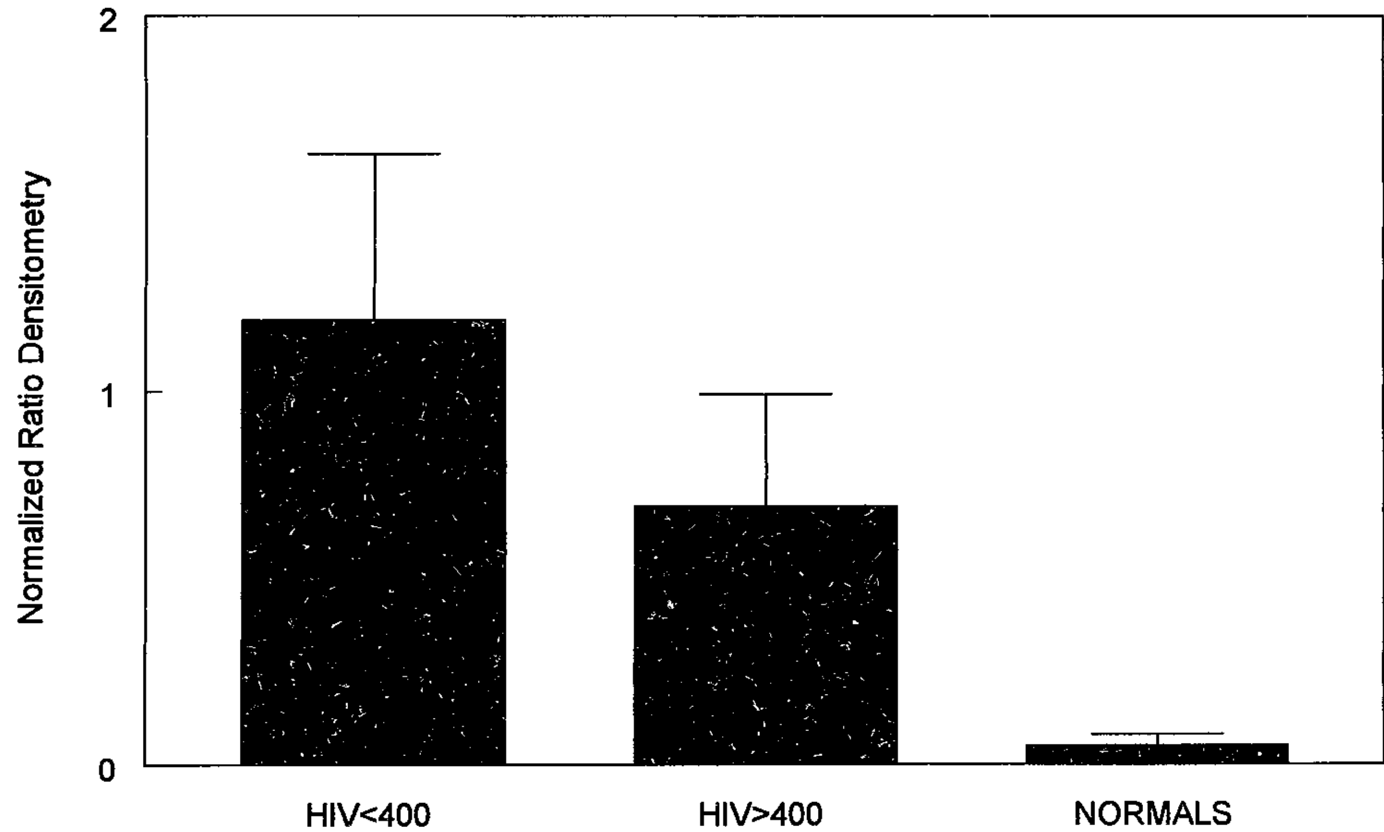
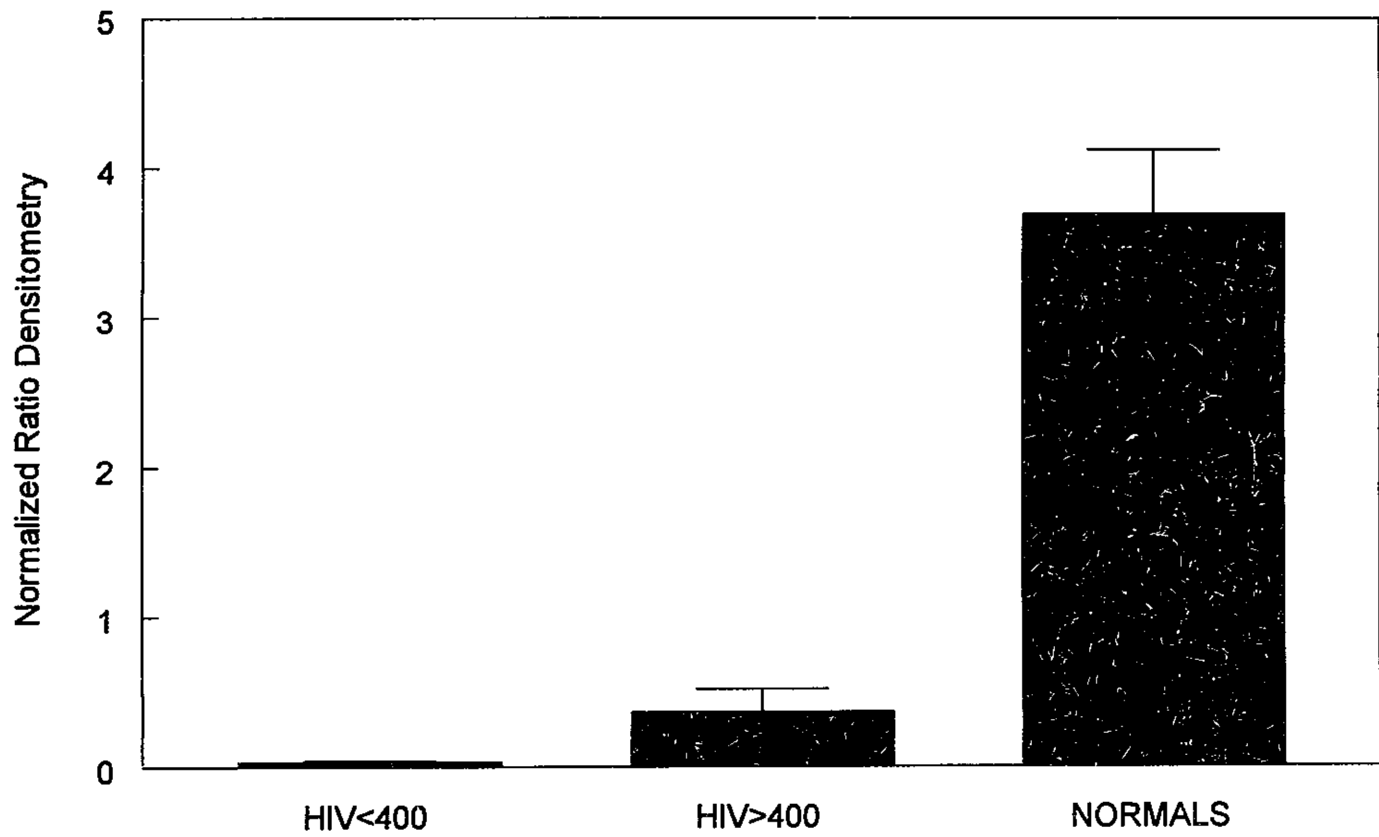

Normalized Ratio Densitometry
2
1
0
HIV<400
HIV>400
NORMALS

FIGURE 10 - IFNγ Semi-quantitative RT-PCR. IFNγ PCR amplified bands as demonstrated by autoradiography were analyzed by densitometry and assigned arbitrary units. The values representing the amounts of amplified IFNγ cDNA was normalized with respect to values for amplified amounts of β-actin cDNA. The black bars represent the mean average of IFNγ normalized densitometry readings of individuals with various $CD4^+$ counts. The cap lines on the graphs represent error bars.


Normalized Ratio Densitometry
5
4
3
2
1
0
HIV<400
HIV>400
NORMALS

Each of the eleven HIV sero-positive patients had less than 400 $CD4^+$ T cell counts. Densitometry scanning of the autoradiographs demonstrated that the ratio of IL-10 to β-actin from HIV sero-positive patients (5.464 ± 2.633) was approximately twenty fold-higher than normal controls (0.310 ± 0.151, $p<0.039$) (Fig.12). In a third data set, PBLs from 14 HIV sero-positive patients and four normal controls PBLs were similarly examined (Fig.13-15). The IL-10 RT-PCR results of 35 HIV sero-positive patients and 15 sero-negative control normals are summarized in Figure 16.

Reproducibility Test

In order to show the reproducibility of RT-PCR in the laboratory, IL-10 RT-PCR was repeated four times using an equal quantity of RNA isolated from four normal individuals. Densitometry scanning of the autoradiogragraphs gave the following readings. The mean values were; normal #11 was (0.3788 ± 0.231), normal #12 was (0.3301 ± 0.212), normal #13 was (0.2282 ± 0.150),and normal #14 was (0.2038 ± 0.1698). Actual densitometry readings were normalized assuming the highest reading(the positive control, PHA stimulated PBLs) to be equal to 1. The results are shown in Figure 17.

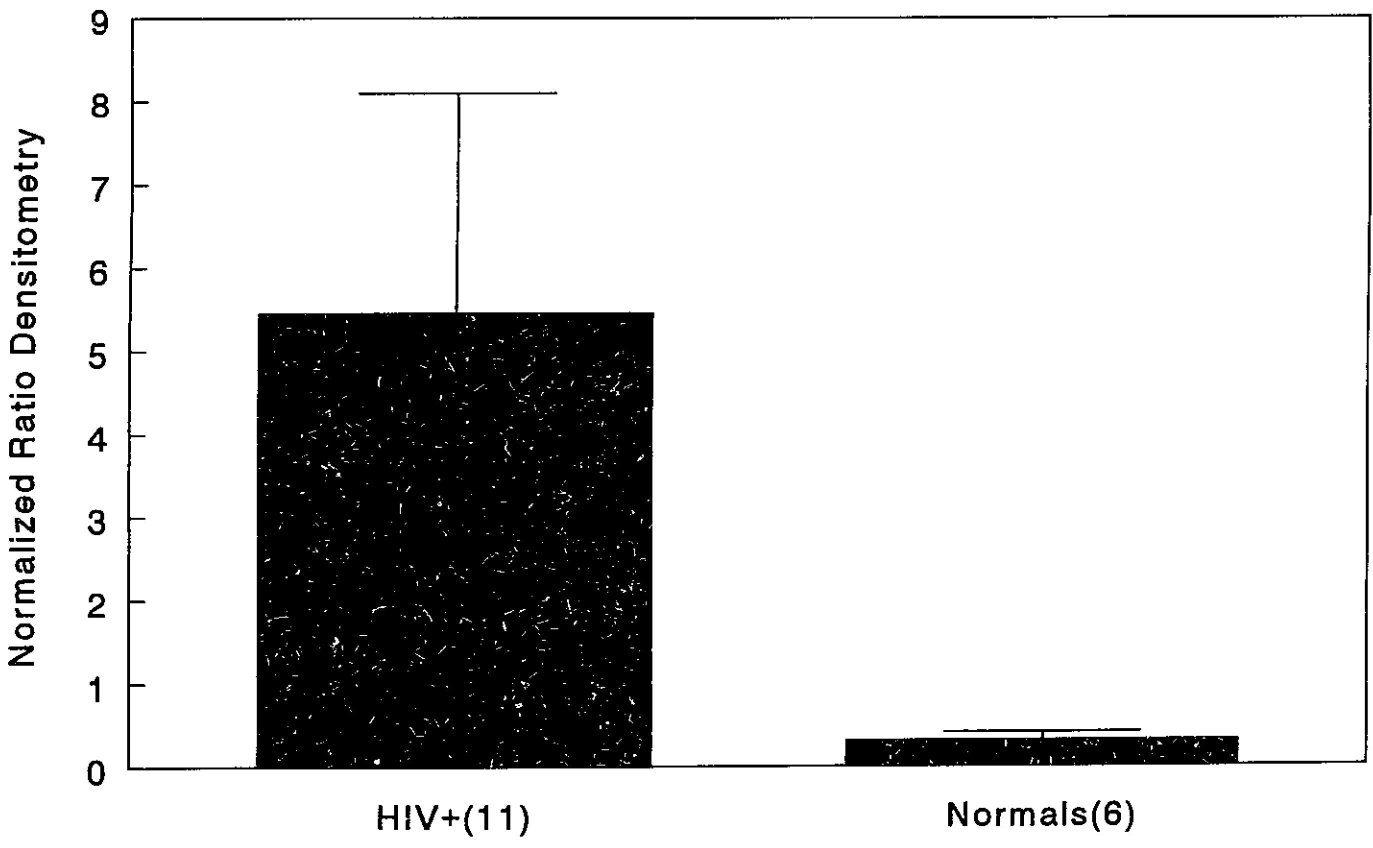
FIGURE 11 - Southern Transfer of IL-10 and β-actin RT-PCR of HIV Immunosuppressed and Normal Individuals. Total RNA from eleven HIV sero-positive patients and six HIV sero-negative individuals were reverse transcribed. Equal amounts of cDNA from each individual were amplified using oligonucleotide primers for IL-10 and β-actin. PCR amplified products were electrophoresed on agarose gel and analyzed by hybridization using probes specific for IL-10 and β-actin. Radioactive blots were exposed to X-ray films. C6 to C11 denote HIV sero-negative individuals. P11 to P21 denote HIV sero-positive patients.

C_6 C_7 C_8 C_9 C_{10} C_{11} P_{11} P_{12} P_{13} P_{14} P_{15} P_{16} P_{17} P_{18} P_{19} P_{20} P_{21}

IL-10 →

ß ACTIN →

FIGURE 12 - IL-10 Semi-quantitative RT-PCR. IL-10 PCR amplified bands as demonstrated by autoradiography were analyzed by densitometry and assigned arbitrary units. The values representing the amount of amplified IL-10 cDNA was normalized with respect to values for amplified amounts of β-actin cDNA. The black bars represent the mean average of IL-10 normalized densitometry readings of HIV sero-positive and normal individuals. The cap lines on the graphs represent error bars.



Normalized Ratio Densitometry
0
1
2
3
4
5
6
7
8
9
HIV+(11)
Normals(6)

FIGURE 13 - Southern Transfer of IL-10, IFNγ, and β-actin RT-PCR of HIV Immunosuppressed and Normal Individuals. Total RNA from fourteen HIV sero-positive patients and four HIV sero-negative individuals were reverse transcribed. Equal amounts of cDNA from each individual were amplified using oligonucleotide primers for IL-10, IFNγ, and β-actin. PCR amplified products were electrophoresed on agarose gel and analyzed by hybridization using probes specific for IL-10, IFNγ, and β-actin. Radioactive blots were exposed to X-ray films. C12 to C15 denote HIV sero-negative individuals. P22 to P35 denote HIV sero-positive patients.

C_{12} C_{13} C_{14} C_{15} P_{22} P_{23} P_{24} P_{25} P_{26} P_{27} P_{28} P_{29} P_{30} P_{31} P_{32} P_{33} P_{34} P_{35}

IFN γ →

IL-10 →

β ACTIN →

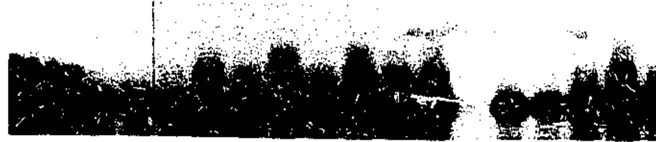


FIGURE 14 - IL-10 Semi-quantitative RT-PCR. IL-10 PCR amplified bands as demonstrated by autoradiography were analyzed by densitometry and assigned arbitrary units. The values representing the amount of amplified IL-10 cDNA was normalized with respect to values for amplified amounts of β-actin cDNA. The black bars represent the mean average of IL-10 normalized densitometry readings of HIV sero-positive and normal individuals. The cap lines on the graphs represent error bars.

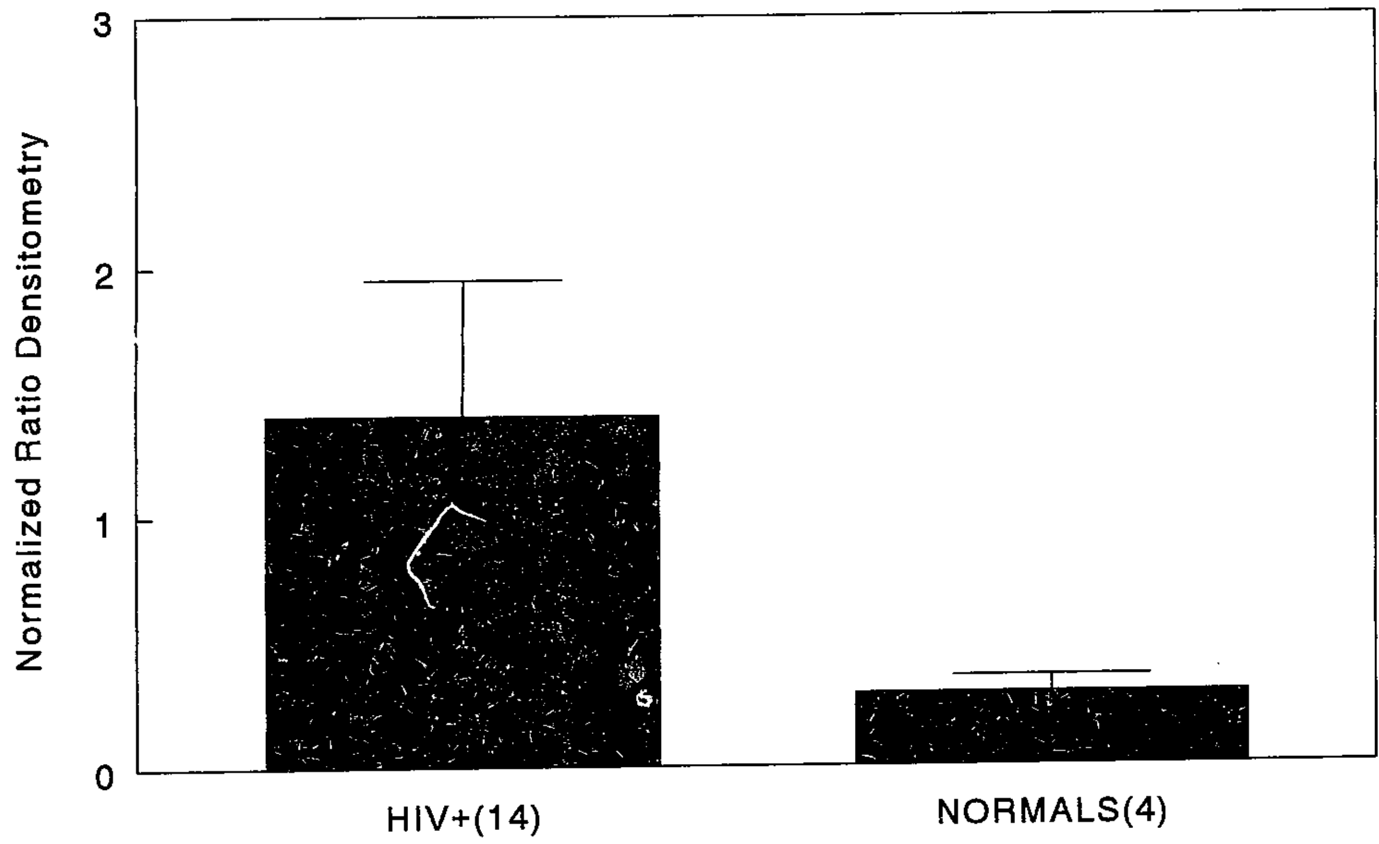

Normalized Ratio Densitometry
3
2
1
0
HIV+(14)
NORMALS(4)

FIGURE 15 - IFNγ Semi-quantitative RT-PCR. IFNγ PCR amplified bands as demonstrated by autoradiography were analyzed by densitometry and assigned arbitrary units. The values representing the amounts of amplified IFNγ cDNA was normalized with respect to values for amplified amounts of β-actin cDNA. The black bars represent the mean average of IFNγ normalized densitometry readings of HIV sero-positive and normal individuals. The cap lines on the graphs represent error bars.

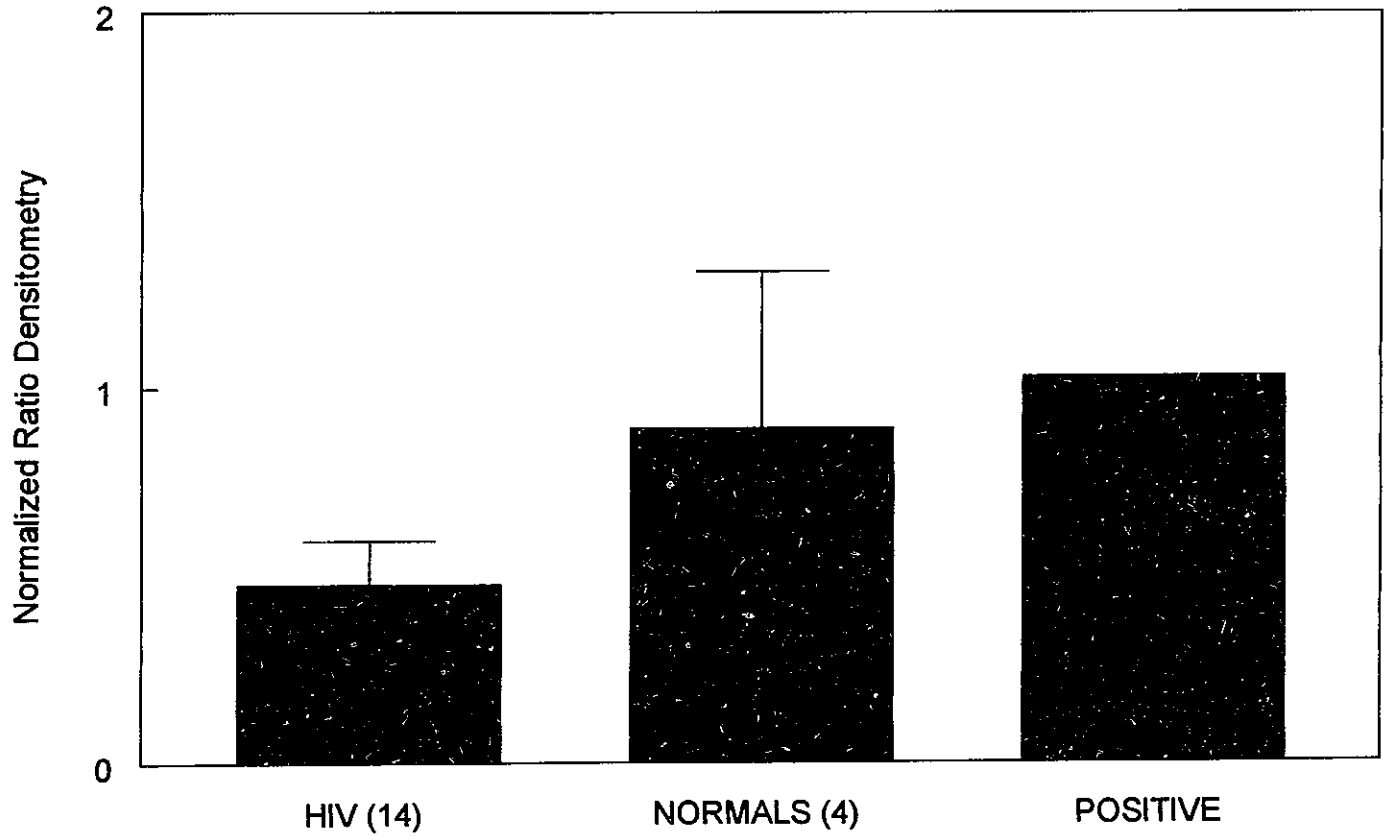

Normalized Ratio Densitometry
2
1
0
HIV (14)
NORMALS (4)
POSITIVE

FIGURE 16 - IL-10 Semi-quantitative RT-PCR for 34 HIV sero-positive and 15 sero-negative individuals. IL-10 PCR amplified bands as demonstrated by autoradiography were analyzed by densitometry and assigned arbitrary units. The values representing the amount of amplified IL-10 cDNA was normalized with respect to values for amplified amounts of β-actin cDNA. The black bars represent the mean average of IL-10 normalized densitometry readings of the total of 50 individuals. The cap lines on the graphs represent error bars.

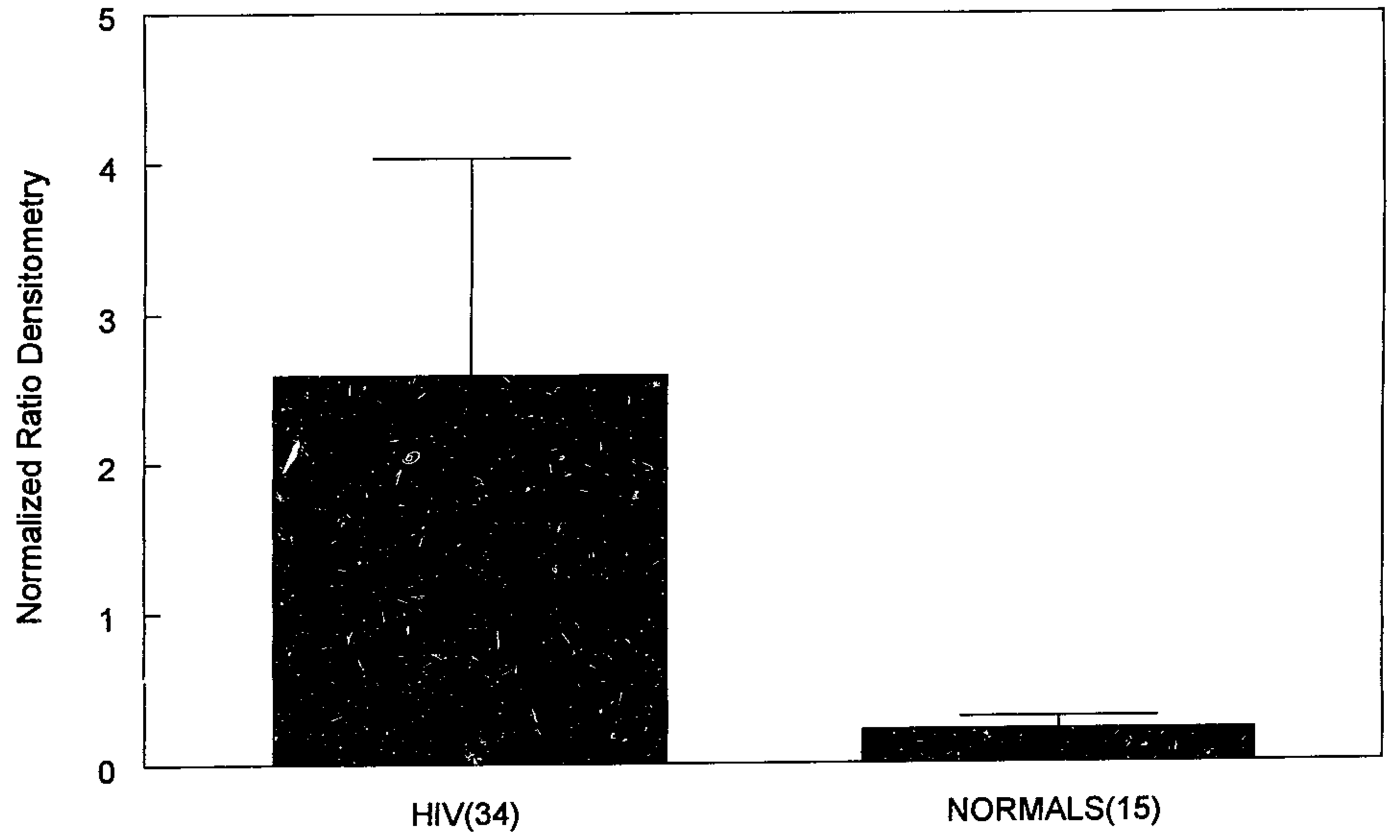
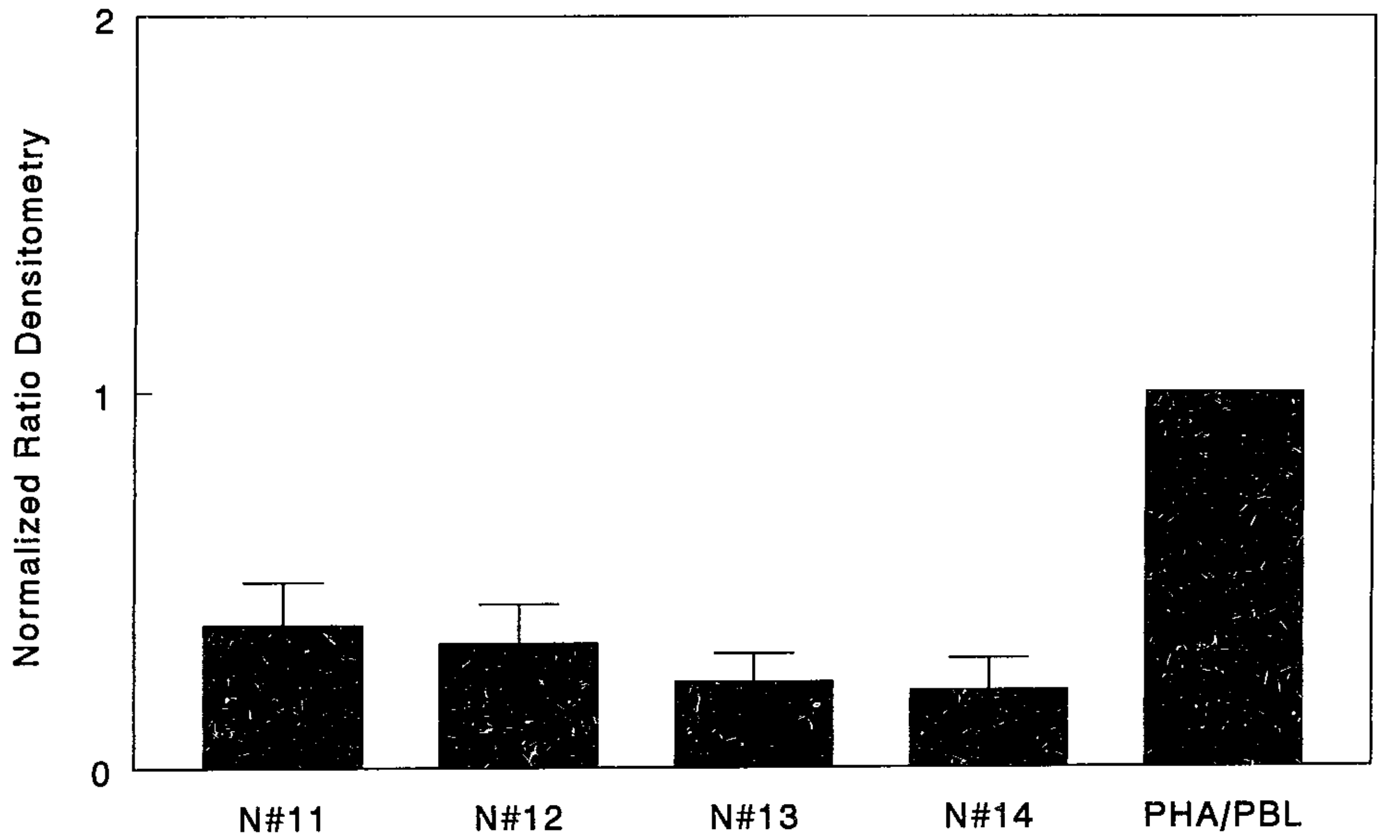
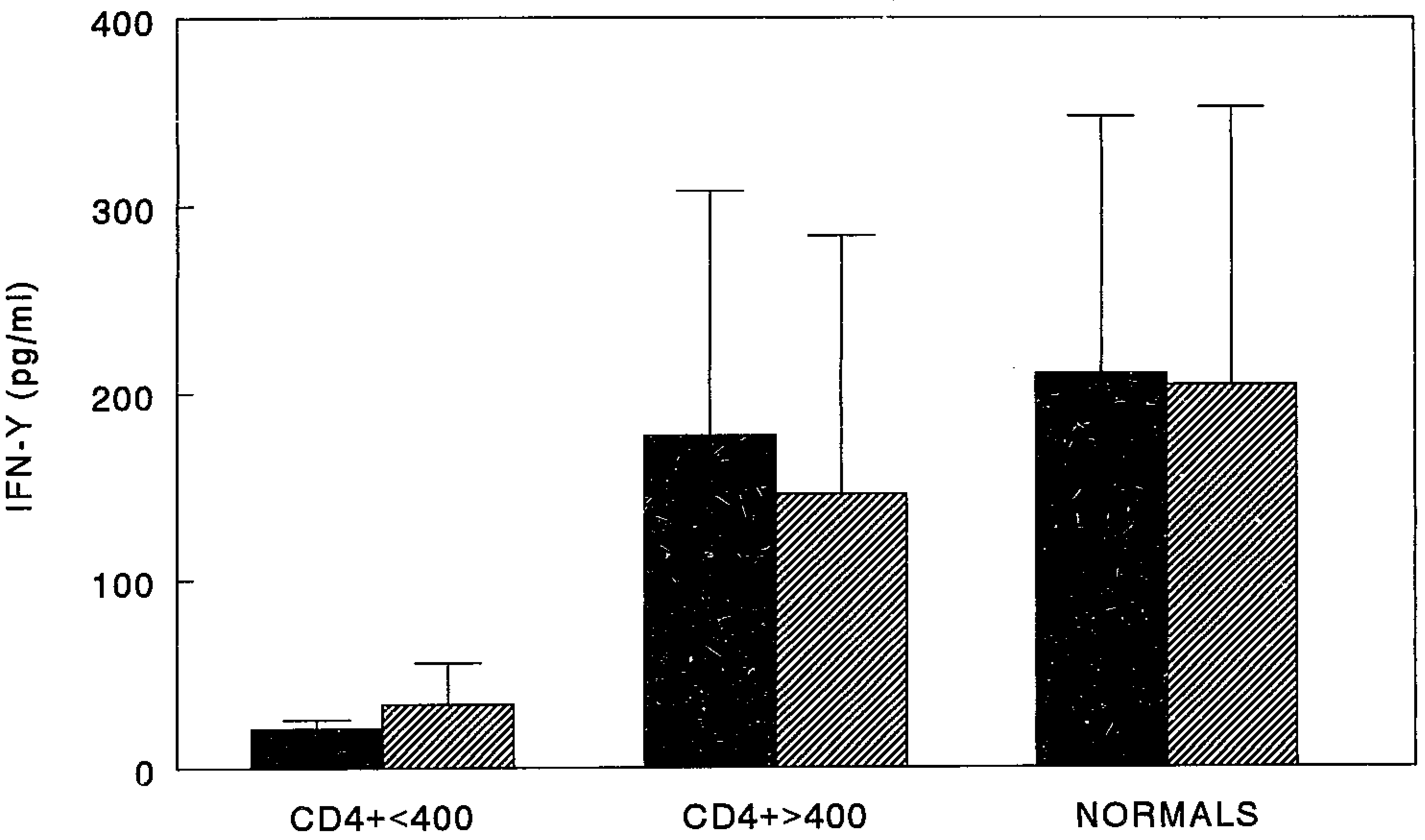

Normalized Ratio Densitometry
5
4
3
2
1
0
HIV(34)
NORMALS(15)

FIGURE 17 - Reproducibility of IL-10 Semi-quantitative RT-PCR. Total RNA from PBLs of four normal individuals were reverse transcribed. Equal amounts of cDNA were amplified for IL-10 at four different times under similar conditions to examine the reproducibility of RT-PCR. PCR amplified products were electrophoresed on agarose gel and analyzed by hybridization using a probe specific for IL-10. Radioactive blots were exposed to X-ray films. IL-10 PCR amplified bands were analyzed by densitometry and assigned arbitrary units. The black bars represent the mean average of IL-10 are normalized densitometry readings of individuals. The cap lines on the graphs represent error bars.


Normalized Ratio Densitometry
2
1
0
N#11
N#12
N#13
N#14
PHA/PBL



IFN-Y (pg/ml)
400
300
200
100
0
CD4+<400
CD4+>400
NORMALS

PRODUCTION OF IFNγ BY PBLs FROM HIV-INFECTED INDIVIDUALS

PBLs obtained from HIV-seropositive individuals with less than 400 $CD4^+$ or with more than 400 $CD4^+$ T cells and from normal controls were stimulated with PHA or anti-CD3 monoclonal antibodies and the supernatants collected 48 hours later were assayed for IFNγ by sandwich ELISA. The amount of IFNγ produced by PBLs stimulated with PHA from patients with more than 400 $CD4^+$ T cells (177 ± 131 pg/ml) was similar to that produced by PHA activated PBLs from normal individuals (210 ± 138 pg/ml). However, patients with less than 400 $CD4^+$ T cells produced significantly lower amounts of IFNγ (21 ± 5 pg/ml; $p<0.001$) as compared to the controls (Fig.18). Similar results for IFNγ production were obtained following activation of PBLs from normal and HIV-infected individuals with anti-CD3 monoclonal antibodies ;normal,204 ± 149 pg/ml; HIV+ patients with >400 $CD4^+$ T cells, 146 ± 138 pg/m; HIV+ patients with <400 $CD4^+$ T cells, 34 ± 22 pg/ml. p= <0.001 (Table 4). Moreover, unstimulated PBLs from HIV-infected individuals had reduced ability to produce IFNγ as measured in the supernatants of unstimulated PBLs (HIV+ patients, 17 ± 13 pg/ml as compared to controls 55 ± 63 pg/ml; $p<0.05$).

FIGURE 18 - Production of IFNγ by PHA and anti-CD3 antibody stimulated PBLs from HIV sero-positive patients with <400 and >400 $CD4^+$ counts and normal individuals. Supernatants of PBL cultures were collected and assayed for IFNγ production by sandwich ELISA. IFNγ levels are indicated in pg/ml. The black bars represent PBLs stimulated with PHA and the crossed bars represent PBLs stimulated with anti-CD3 antibody.

TABLE 3 - IFNγ ELISA reading of stimulated peripheral blood lymphocytes of HIV sero-positive with less than 400 CD4+, more than 400 CD4+, and HIV-

Mitogen	HIV^{+} <400 $CD4^{+}$	HIV^{+} >400 $CD4^{+}$	HIV-
PHA	21	177	210
Anti-CD3	34	146	204

Production of IFNγ by PHA and anti-CD3 antibody stimulated PBLs from HIV infected individuals and normal individuals. Supernatants of PBLs were collected and assayed for IFNγ production by a sandwich ELISA. IFNγ levels are presented as pg/ml units.

CHAPTER 4

Discussion

A significant portion of the knowledge regarding IL-10 has come solely from *in vitro* studies that evaluate its effects on isolated cell populations. Part of the focus of this study was to assess whether these *in vitro* properties accurately reflect the physiological function of IL-10 *in vivo*. HIV sero-positive patients were chosen as the model system for this study since such patients demonstrate immunosuppression, the main effect of IL-10 on the immune response. Also, since hIL-10 and vIL-10 are able to completely suppress the specific proliferative responses of $CD4^+$ T cells and $CD4^+$ T cell clones towards various protein antigens and antigenic peptides (de Waal Malefyt, 1991b) and HIV sero-positive patients are known to have reduced counts of $CD4^+$, studying such a model system could reveal new information regarding the role of IL-10 in the cytokine network. Such information could potentially provide a better understanding of the status of immunosuppression due to IL-10 in immunodeficient individuals such as AIDS patients.

The cellular basis for the gradual immunodeficiency in HIV infection is not well understood. Gradual loss of proliferative responses to recall antigens and lectins observed

with disease progression may be credited to the disruption of cytokine cross-regulation (Clerici, 1993; Clerici, 1989). Immunosuppression induced by HIV proteins such as gp 120, nef and tat proteins has been ascribed to interference with signal transduction and cytokine production (Weinhold, 1989; Viscidi, 1989). Non-responsiveness to antigens in the setting of HIV infection usually results from impaired IL-2 production and can be reversed by its exogenous administration (Clerici, 1993; Clerici, 1989). Selective loss of IL-2 production is further correlated with the loss of IFNγ production and a corresponding increase in IL-4 production (Rosenberg, 1990; Maggie, 1987) suggesting up-regulation of Th2 type and down-regulation of Th1 type responses. Furthermore, Th2 type responses such as B cell hyperplasia, enhanced levels of IgG and IgE in serum and B cell lymphomas are frequently observed in HIV infection (Rosenberg, 1990; Schnittman, 1986) resulting from exaggerated humoral responses at the cost of suppressed cell mediated responses. These observations suggest that as HIV disease progresses, a selective dysfunction of Th1 and upregulation of Th2 class responses may occur.

The relative expression of cytokines produced preferentially by Th1 and Th2 type cells (namely IFNγ and IL-10 respectively) were analyzed based on the hypothesis that HIV infection may preferentially downregulate $CD4^+$ T cells with Th1 cytokine secretion patterns. Unlike controls, PBLs from HIV-seropositive patients, before and after activation with PHA and anti-CD3 monoclonal antibodies, produced significantly lower amounts of IFNγ as assessed by both a biological assay and semi-quantitative RT-PCR. In addition, decreased production of IFNγ was inversely correlated with increased expression of IL-10. These observations suggest that the dominance of cytokines,

especially a Th2 class response, may potentiate the negative regulation of HIV infection on Th1 cell proliferation and cytokine synthesis. Our main method of choice, reverse transcriptase polymerase chain reaction, has innate limitations. The efficiency for the accuracy of the cDNA synthesis step is variable between methods, and ranges from 5% to 90% (Noonan, 1990; Henrard, 1992; Simmonds, 1990). In addition, the ability of polymerase chain reaction to amplify small amounts of nucleic acids, exponentially is also the factor that makes the technique challenging as a quantitative method. Any element that is capable of interfering with the exponential amplification might drastically decrease the intrinsic quantitative ability of PCR (Mullis, 1991).

Immunoregulatory cytokines play an important role in replication of HIV in $CD4^+$ T cells as well as in cells of the monocyte/macrophage system. HIV-inductive cytokines like TNF-α, IL-6, and GM-CSF trigger replication of HIV by activating the transcription factor NFκB which subsequently binds in the enhancer region of the HIV proviral LTR (Folks, 1989; Poli, 1990; Osborn, 1989; Howard, 1993; Hammer, 1986; Koyanagi, 1988). In contrast, transforming growth factor B, IFN-α, and IFN-β have been shown to suppress viral replication (Poli, 1991; Poli, 1992; Kornbluth, 1989). However, IFNγ exerts dichotomous effects on HIV replication depending on experimental conditions. It directly enhances viral replication in the U1 promonocytic cell line but prevents its replication following PMA treatment (Koyanagi, 1988; Poli 1992; Kornbluth, 1989). It has been shown that IFNγ expression is reduced significantly in both unactivated and polyclonally activated PBL from HIV infected patients (Koyanagi, 1988; Gendelman, 1990; Murray, 1988). This is apparently the first report describing IL-10 upregulation and its inverse

correlation with IFNγ expression in unstimulated PBLs of HIV seropositive patients. It was found that there was an increase in the expression of IL-10, and that there is an inverse correlation between the expression of IFNγ and IL-10 in this population. Il-10 up-regulation and the consequent IFNγ downregulation may influence innate immune responses and their effects on progression of disease. Human IL-10 is produced by both Th1 and Th2 cell types although preferentially by Th2 cells (Del Prete, 1993). IL-10 up-regulation in B cell lymphomas from HIV patients has been reported but was attributed to EBV transformation (Burdin, 1993; Emilie, 1992; Benjamin, 1992). The patients in the present study exhibited neither B cell lymphomas nor infectious mononucleosis. It has been shown that patients with high levels of EBV replication had an increased risk of HIV disease progression (Diaz-Mitoma, 1990). It is not known whether there is a direct correlation between high EBV loads and IL-10 expression in HIV infection, but over-expression of IL-10 in this setting may contribute to the observed B cell hyperplasia and hypergammaglobulinemia owing to its activity as a B cell growth factor (Rousset, 1992; Go, 1990). IL-10 may also contribute to the defective antigen/recall response in HIV infection due to its down-regulatory effects on IFNγ production, major histocompatibility class II molecule expression and antigen presentation (de Waal Malefyt, 1991; Howard, 1992). Furthermore, over-expression of IL-10 in AIDS patients raises the fundamental question of the effect of IL-10 on HIV replication.

Results of these studies also suggests that expression of cytokines with presumably protective effects could be modulated. IFNγ production by PHA-activated PBL from AIDS patients was downregulated by IL-10. A possible explanation could be the

selective loss of IFNγ producing Th1 cells. Another possible explanation would be that HIV infected macrophages may produce certain factors such as IL-12 (Hsieh, 1993; Manetti, 1993) which either alone or in conjunction with IL-10 may prevent IFNγ downregulation.

Studies were initiated to elucidate the expression and regulation of IL-10 in the PBLs of HIV sero-positive patients assuming that HIV induces selective loss or dysfunction of memory T cells with Th1-like function, derepression of IL-10 expression, and predominance of Th2-like cytokine production (Clerici, 1993; Shearer, 1992). HIV may also modulate IL-12 expression, either directly after infection of monocytes or indirectly via IL-10. This dysregulation may partially explain, the selective defect in cell-mediated immunity and the predominance of humoral immune responses observed in HIV sero-positive patients.

Macrophage activation by different stimuli, including IFNγ, is blocked by IL-10 (Moore, 1993). The cDNA encoding IL-10 receptor is a member of the interferon receptor (IFNR)-like subgroup of the cytokine receptor (CR) family (Suk Yue HO, 1993). The observed relationship of IL-10R and IFNγR suggests several possibilities. First, because of observations regarding shared subunits among receptors for different cytokines (Miyajima, 1992), it is possible that IL-10R could likewise share a second receptor chain with an IFNR as has been shown. If this is true, IL-10 and IFNγ might compete for binding to each other's receptor. A second theory is that IL-10R activation might directly antagonize the IFNR signal transduction pathway (Shuai, 1992; Sen, 1992), possibly by interacting with one or more intracellular components.

The objectives in this study were 1) to produce biologically active hIL-10 and vIL-10, which were not commercially available at the time, in order to develop IL-10 biological assays 2) to establish a semi-quantitative RT-PCR in order to detect, semi-quantitatively measure, and compare IL-10 to IFNγ in immunodeficient HIV sero-positive patients and normal controls; both of which were accomplished. In conclusion, there is evidence to suggest that HIV specifically inhibits the cytokines responsible for the cell-mediated immune responses with consequent upregulation of cytokines mediating humoral responses. HIV induces cytokines that facilitate its own replication. However, in contrast, HIV upregulates cytokines like IL-10 which may act in a paracrine manner to downregulate protective cytokines like IFNγ as well as the proliferation of Th1 cells responsible for cell-mediated immune response (CMIR). Modulation in the expression of immunoregulatory cytokines like IL-2, IL-10, and IFNγ (Hsieh, 1993; Manetti, 1993) by therapeutic intervention thus raises the possibility of devising means to generate protective immune responses against HIV.

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