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Expression of IL-10, IL-4 and interferon-gamma in unstimulated and mitogen-stimulated peripheral blood lymphocytes from HIV-seropositive patients

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SUMMARY

Infection of immune cells with HIV induces dysregulation of cytokines which may play a vital role in HIV pathogenesis. We analysed the expression of T helper type 1 (Th1) (interferon-gamma (IFN-γ)) and Th2 (IL-4, IL-10) type cytokines in peripheral blood lymphocytes (PBL) from HIV$^+$ patients. The semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that IFN-γ mRNA in unstimulated PBL was significantly decreased and IL-10 mRNA was significantly upregulated in patients with <400 CD4$^+$ T cells/mm^3 ($n=30$) as compared to patients with >400 CD4$^+$ T cells/mm^3 ($n=6$) and normal controls ($n=16$). In addition, IL-10 mRNA levels were inversely associated with IFN-γ expression. Similar results were obtained by measuring IL-10 production in the supernatants of PBL cultured *in vitro* without stimulation by employing an enzyme immunosorbent assay (ELISA). However, the levels of IL-4 and IFN-γ produced by unstimulated PBL were undetectable by ELISA. Mitogen stimulation of PBL revealed two groups of HIV$^+$ individuals based on IL-10 production. PBL from one set of individuals produced low levels of IL-10 (low IL-10 producers) whereas the other group produced IL-10 comparable to that of normal controls (IL-10 producers). Production of IL-4 was significantly reduced in HIV$^+$ individuals with <400 CD4$^+$ T cells/mm^3 as compared to the normal controls. However, ability to produce IFN-γ by mitogen-stimulated total PBL and CD4$^+$ purified cells was not impaired in HIV$^+$ individuals. These results suggest that unstimulated and mitogen-stimulated PBL of HIV$^+$ individuals exhibit dysregulation of Th2 type cytokines which may play a role in HIV immunopathogenesis.

Keywords IL-10 IL-4 interferon-gamma HIV

INTRODUCTION

The complex interaction between HIV and host immune cells results in a state of profound immunodeficiency [1]. Following infection, immune defects including sequential loss of responsiveness to recall antigens, alloantigens and mitogens are observed [2,3]. A number of qualitative changes are also observed such as the disruption of the CD4–MHC complex, defective signal transduction, disruption of the normal cytokine network and a direct cytopathic effect on CD4$^+$ T cells [4]. In addition, infection of host T cells and macrophages with HIV induces the expression of cytokines such as tumour necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6, which directly enhance viral replication in HIV$^+$ cells *in vitro* [1,5,6].

In addition to the direct loss of CD4$^+$ T cells, immunosuppression may also result from the release of cytokines which inhibit the expression of growth factors essential for normal functions of immune effector cells. T helper type 2 (Th2) cells, B cells and macrophages in both mouse and man have been shown to secrete IL-10, a cytokine synthesis inhibitory factor which inhibits essential elements of the anti-viral response, including interferon-gamma (IFN-γ) and IL-2 synthesis by Th1 cells [7–11]. IL-10 is a pleiotropic molecule possessing a broad spectrum of biological activities. It is a growth factor for normal and Epstein-Barr virus (EBV) transformed human B cells and a promoter of proliferation of IL-2 activated cytotoxic T cells [7–11]. IL-10 may also contribute to the polyclonal B cell proliferation and hypergammaglobulinaemia observed in

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HIV$^+$ patients [1] as suggested by *in vitro* studies [9]. Moreover, the EBV-induced B cell lymphomas frequently observed in HIV infection exhibit enhanced IL-10 synthesis [10,11] which may further aggravate immunosuppression. These observations suggest that IL-10 is intimately associated with the pathogenesis of HIV. Similarly, enhanced expression of IL-4, a Th2 type cytokine, has been documented [2,12]. It has been hypothesized that Th2 class responses may predominate in HIV infections [2,12]. IL-10 levels were found to be significantly elevated in phytohaemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBL) of HIV$^+$ individuals as compared to healthy controls [13]. Furthermore, IL-10 upregulation in these cells was correlated with the severity of disease [13]. However, levels of *in vivo* expression of the immunoregulatory cytokines IL-10 and IFN-γ in PBL are not known. Furthermore, conflicting results have recently been obtained regarding the Th1–Th2 switch hypothesis in HIV disease progression [14–18]. To study the relative levels of T helper class responses, we measured IL-10, IL-4 and IFN-γ *in vivo* and *in vitro* in the unstimulated and mitogenically stimulated PBL of HIV$^+$ patients. Our results do not suggest the preferential expression of Th2-type cytokines in HIV$^+$ individuals.

MATERIALS AND METHODS

Isolation and culture of peripheral blood lymphocytes

Blood was obtained for mononuclear cell isolation from healthy adult volunteers, HIV$^+$ individuals with <400 CD4$^+$ T cells/mm^3 and HIV$^+$ individuals with >400 CD4$^+$ T cells/mm^3. Blood samples were collected following approval of the protocol by the Ethics Review Committee of the Ottawa General Hospital, University of Ottawa, Ottawa, Ontario, Canada. Clinical information for each of the HIV$^+$ patients including CD4$^+$ and CD8$^+$ T cell counts was also obtained. All patients were asymptomatic and were EBV seropositive but had no clinical manifestation of infectious mononucleosis or of B cell lymphomas. None of the patients had clinical evidence of bacterial or fungal infection at the time of specimen collection. Standard methods were used for cell preparation and fractionation [19]. In brief, PBL were isolated by density gradient centrifugation using Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). The cell layer consisting mainly of mononuclear cells was collected and washed three times in Hanks' buffered salt solution (HBSS). The PBL thus obtained were either frozen for measurement of cytokine mRNA specific for IL-10 and IFN-γ by reverse transcription-based polymerase chain-reaction (RT-PCR) or resuspended in complete RPMI-1640 medium. Complete medium consisted of RPM1-1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 mM HEPES and 2 mM glutamine.

Measurement of IL-10, IL-4 and IFN-γ by enzyme linked immunosorbent assay (ELISA)

To determine the ability of PBL from HIV$^+$ individuals to produce cytokines, the cells were cultured at a concentration of 2×10^6 cells/ml in 24-well tissue-culture plates (Falcon Labware, Oxnard, CA) in the presence and absence of phytohaemagglutinin-M (PHA, final dilution 1:50) (GIBCO) or anti-CD3 antibodies (1:200 final dilution of cell supernatant). Anti-CD3 antibody hybridoma (CRL 8001) was kindly provided by the American Tissue Culture Collection (ATCC, Rockville, MD). The supernatants were harvested after 24, 48 and 72 h of stimulation and were frozen at −70°C.

IFN-γ. A sandwich ELISA was employed to quantify the IFN-γ produced by PBL. Two different mouse monoclonal antibodies (MoAb) recognizing two distinct IFN-γ epitopes were used. MoAb γ3-11.1 (ATCC) was used at a concentration of 5 μg/ml in PBS for coating the high efficiency binding plates (Nunc Immunomodules, Denmark) at 4°C overnight. The plates were blocked with 5% skimmed milk in PBS–Tween 20 (0·05%). IFN-γ was detected by a second MoAb B-24 (UBI-Olympus, Lake Success, NY) which was biotinylated as described [20] at a concentration of 5 μg/ml in PBS–Tween 20. Streptavidin conjugated with horseradish peroxidase (Jackson Immuno-Research, West Grove, PA) was used at a final dilution of 1:1000. The colour reaction was developed by *o*-phenylenediamine dihydrochloride (OPD) (Sigma, St Louis, MO) and hydrogen peroxide which was measured at 540 nm. Recombinant IFN-γ (BioSource International, Camarillo, CA) was used concurrently as a standard. IFN-γ was detectable at greater than 16 pg/ml in this assay.

IL-10. IL-10 was measured by a sandwich ELISA using two different MoAbs which recognize two distinct epitopes as described previously [21]. Briefly, the plates (Nunc Immunomodules) were coated overnight at 4°C at a concentration of 3 μg/ml of purified anti-human and viral IL-10 MoAb, JES3-9D7 (rat IgG1 obtained from PharMingen, San Diego, CA) in the coating buffer (0·1 M $NaHCO_3$, pH 8·2). The plates were washed with PBS–Tween 20 and blocked with PBS–10% FCS. IL-10 was detected by employing a second biotinylated MoAb, 18562D (rat IgG2a obtained from PharMingen) at a concentration of 3 μg/ml in PBS–10% FCS. Streptavidin peroxidase (Jackson Immuno-Research) was used at a final dilution of 1:1000. The colour reaction was developed by OPD and hydrogen peroxide. Recombinant IL-10 (R&D Systems, Minneapolis, MN) was used as a standard. The sensitivity of the IL-10 ELISA was 16 pg/ml. IL-10 stimulation index (SI) was calculated as a ratio of IL-10 levels (pg/ml) obtained following stimulation of PBL with mitogens to the IL-10 levels produced by unstimulated PBL.

IL-4. IL-4 was quantified by sandwich enzyme immunoassay using the human IL-4 Quantikine^{TM} kit (R&D Systems) essentially as described by the manufacturer. Recombinant human IL-4 provided in the kit was used as a standard. IL-4 was measured in the culture supernatants harvested 48 h after stimulation with PHA. The sensitivity of the assay was 16 pg/ml.

Isolation of CD4$^+$ cells from PBL of HIV$^+$ and normal individuals

CD4$^+$ cells were isolated from PBL by immunomagnetic beads conjugated with anti-CD4 antibodies as described by the manufacturer (Dynal A.S., Oslo, Norway). Briefly, PBL were washed three times with PBS and resuspended in PBS at a concentration of 50×10^6 cells/ml. PBL were incubated with anti-CD4 antibody conjugated immunobeads (Dynabeads, M-450, Dynal) at a bead to target cell ratio of 20:1 at 4°C for 30 min. The cells attached to the beads were washed six times with PBS containing 2% fetal calf serum (FCS) and subsequently resuspended in IMDM containing 1% FCS at 2×10^6

cells/ml. The purified $CD4^+$ cells bound to the immunobeads (1×10^6 cells/ml) were stimulated with PHA (final dilution, 1 : 50) for 48 h. The cells were harvested and frozen at −70°C for subsequent isolation of RNA and RT-PCR analysis. The presence of immunobeads neither inhibited T cell stimulation as determined by ^{3}H-thymidine incorporation nor had any effect on the quality of isolated RNA (data not shown).

RNA isolation and detection of IL-10 and IFN-γ mRNAs

Total cellular RNA was extracted from PBL obtained from both normal and HIV^+ individuals using a monophase solution containing guanidine isothiocyanate and phenol (Tri Reagent solution, Molecular Research Center, Inc., Cincinnati, OH) as described by the manufacturer. The RT-PCR technique was used to assess levels of IL-10 and IFN-γ mRNA expression. cDNAs were generated by reverse transcription from 1 μg of total RNA using the Perkin Elmer thermal cycler 9600 and Perkin Elmer Gene Amp RT-PCR kit (Perkin Elmer, Norwalk, CT) as described by the manufacturers. Briefly, the reverse transcription was performed using 2·5 mM $MgCl_2$, 1 mM each of the nucleotides (dNTP), 1 U/μl RNase inhibitor and 2·5 μM random hexamers in a final 10 μl volume. The reaction was carried out under the following conditions: 42°C for 15 min followed by 94°C for 5 min and 5°C for 5 min. Equal aliquots of cDNA were subsequently amplified for IL-10, IFN-γ and β-actin using 2·5 units of AmpliTaq DNA polymerase, 150 pM each of the appropriate 5′ and 3′ primers, 1 mM of each dNTP and 4 mM $MgCl_2$ in a total volume of 100 μl. The mixture was subjected to 35 cycles of DNA amplification. The oligonucleotide primer sequences for IL-10, IFN-γ and β-actin are listed below and the conditions for amplification were optimized for each of them. For IL-10, the first cycle consisted of denaturing at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 1 min and primer annealing and extension at 60°C for 1 min. The last extension was at 60°C for 7 min. For IFN-γ and β-actin, the first cycle consisted of denaturation at 94°C for 5 min followed by primer annealing at 60°C for 5 min. The PCR reaction consisted of 30 cycles for β-actin and 35 cycles for IFN-γ, with denaturation at 95°C for 45 s followed by primer annealing at 60°C for 45 s and extension at 72°C for 1·5 min. Finally, the last cycle was at 72°C for 10 min. PCR products (20 μl) were resolved by electrophoresis on 2% agarose gels. To ensure the detection of specific amplified cDNA, the PCR products were transferred onto Zeta probe filters (BioRad Laboratories, Richmond, CA) according to manufacturer's specifications. The filters were then hybridized to ^{32}P-labelled cDNA probes specific for the amplified products (Prime IT II kit, Stratagene, La Jolla, CA).

Primers and probes

The following primers for IL-10, IFN-γ and β-actin were used. IL-10: sense 5′–ACA GGA TCC TAT GCA CAG CTC AGC ACT GCT C–3′; antisense 5′-TAG GAT CCT CAC CTG GCT TTA ATT GTC ATG TAT GC–3′. The primers for IL-10 were synthesized by National Bioscience (Plymouth, MN) which amplified a 531 bp product and were specific for human (not viral) IL-10. Oligonucleotide primers for IFN-γ amplified a PCR product of 501 bp and were provided by Stratagene, which are as follows: sense 5′–ATG AAA TAT ACA AGT TAT ATC TTG GCT TT–3′; antisense 5′–GAT GCT CTT CGA CCT CGA AAC AGC AT–3′. The primers for β-actin were as follows: sense 5′–TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA–3′ and antisense 5′–CTA GAA GCA TTG CGG TGG ACG ATG GAG GG–3′ and amplified a PCR product of 610 bp. The human IL-10 cDNA probe consisted of a Bam H1 fragment of pUC18-IL10 plasmid which was subcloned from pcDSR α-IL-10 (ATCC) using standard techniques [22]. The probes used to detect IFN-γ and β-actin were the PCR products amplified by RT-PCR employing the above mentioned oligonucleotide primers.

Semiquantitative measurement of IL-10 and IFN-γ mRNA by RT-PCR analysis

The quantification of specific mRNA for IL-10 and IFN-γ expression in PBL by semiquantitative RT-PCR analysis involved comparing the signals for IL-10 and IFN-γ with respect to β-actin. Equal amounts of cDNA obtained following reverse transcription of cellular RNA from PBL of patients and normal individuals was PCR amplified for β-actin, IL-10 and IFN-γ. The autoradiographs obtained following hybridization were scanned by densitometry. The signals specific for IFN-γ, IL-10 and β-actin mRNA were measured by densitometry with the help of an Image software program (Image-Pro Plus, Media Cibernetics, Silver Spring, MD). Density values for IL-10 and IFN-γ were provided as a ratio of the cytokine to the β-actin signal to normalize the expression of cytokines to β-actin. The relative expression of IL-10 and IFN-γ was analysed by comparing the ratio of the densitometric units for IL-10 to β-actin, and IFN-γ to β-actin respectively.

Statistical analysis

Means were compared by the two-tailed Student's *t*-test. The results were expressed as mean ± s.e.m. The frequencies of samples below and above the threshold values (16 pg/ml) were compared between the experimental groups using the χ^2 test.

RESULTS

Optimization of semi-quantitative RT-PCR analysis

Since quantification of cytokines involved measuring relative expression of IL-10 and IFN-γ with respect to β-actin, the PCR technique was optimized for reproducibility and accuracy. For this, various concentrations of mRNA obtained from PBL of a normal individual ranging from 25 to 100 ng were reverse transcribed. The cDNA was amplified for 25, 30 and 35 cycles using β-actin primers. Measurement of the signals by densitometric analysis revealed that amplification of β-actin was directly proportional to the amount of cDNA used for amplification. Furthermore, dose-dependent amplification was not observed as the number of amplifying cycles was increased beyond 30 (data not shown). Therefore, in our experiments, we employed 30 amplifying cycles for β-actin PCR analysis. To test reproducibility in the amplification of cDNA, reverse transcribed cDNA from PBL of four HIV^+ individuals and EBV transformed B-95/8 B cells were amplified. Four aliquots from the same sample each containing 25 ng of cDNA were amplified for β-actin and IL-10 at different times under similar conditions. Similar values were obtained following densitometric scanning of IL-10 and β-actin expression signals in the samples going through the same system. Furthermore, the ratios of densitometric values of IL-10 to β-actin expression signals were also similar (Fig. 1).

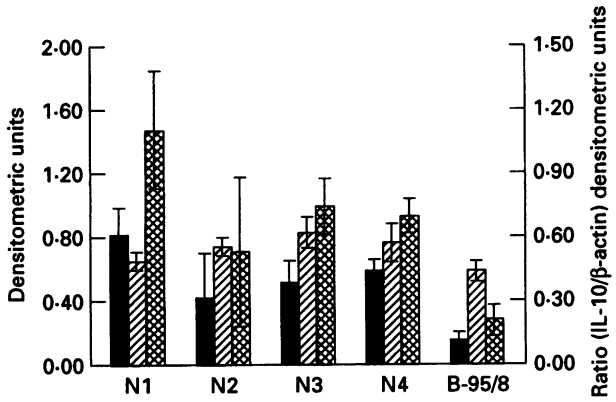

Fig. 1. Reproducibility of semiquantitative RT-PCR analysis. Total RNA from four HIV$^+$ individuals and an EBV-transformed B cell line, B-95/8, were reverse transcribed. Four aliquots of equal amounts of cDNA were amplified for IL-10 and β-actin at four different times under similar conditions. The signals obtained on the autoradiographs for IL-10 and β-actin were scanned by densitometry and the ratio of IL-10 to β-actin was calculated. The results are expressed as the means of values for the four aliquots in each patients ± s.d. ■, IL-10 DU; ▨, β-actin DU; ▩ ratio of IL-10 to β-actin DU.

Downregulation of IFN-γ expression in PBL from HIV$^+$ individuals

The ability to produce IFN-γ by PBL from 30 HIV$^+$ and 16 normal individuals was assessed by subjecting equal amounts of mRNA to semiquantitative RT-PCR analysis for IFN-γ and β-actin. The densitometric scanning of the autoradiographs assigning arbitrary densitometric units (DU) demonstrated that the ratio of IFN-γ to β-actin signals from HIV$^+$ patients with <400 CD4$^+$ T cells (DU = 0·24 ± 0·07) was significantly lower than the normal controls (DU = 1·41 ± 0·60; $P < 0·06$ (Fig. 2b)). Furthermore, the IFN-γ mRNA amplified from PBL from 60% of HIV$^+$ patients with <400 CD4$^+$ T cells was 10-fold lower than the normal controls. Although a trend towards lower levels of IFN-γ was observed in PBL of patients with >400 CD4$^+$ T cells (DU = 0·36 ± 0·15) than in normal controls (DU = 1·41 ± 0·60), the difference was not statistically significant (Fig. 2b).

Upregulation of IL-10 in PBL of HIV$^+$ individuals and downregulation of IFN-γ expression

IFN-γ has been shown to be regulated by IL-10 [7–9]. To study whether reduced expression of IFN-γ in HIV$^+$ patients is associated with enhanced expression of IL-10, PBL obtained from both HIV$^+$ patients and controls were assayed for IL-10 expression by semiquantitative RT-PCR analysis. Equal amounts of cDNA were amplified for IL-10 and β-actin. The ratio of arbitrary units obtained by densitometry from IL-10 and β-actin autoradiographs revealed significantly higher expression of IL-10 in HIV$^+$ patients with <400 CD4$^+$ T cells (DU = 2·82 ± 1·04) than in HIV$^+$ patients with >400 CD4$^+$ T cells (DU = 0·57 ± 0·27, $P < 0·05$) and normal controls (DU = 0·32 ± 0·08; $P < 0·01$ (Fig. 2b)). However, significant differences in the expression of IL-10 in HIV$^+$ individuals with >400 CD4$^+$ T cells (DU = 0·57 ± 0·27) and normal controls (DU = 0·32 ± 0·08) were not observed. An inverse correlation in the expression of IL-10 and IFN-γ was

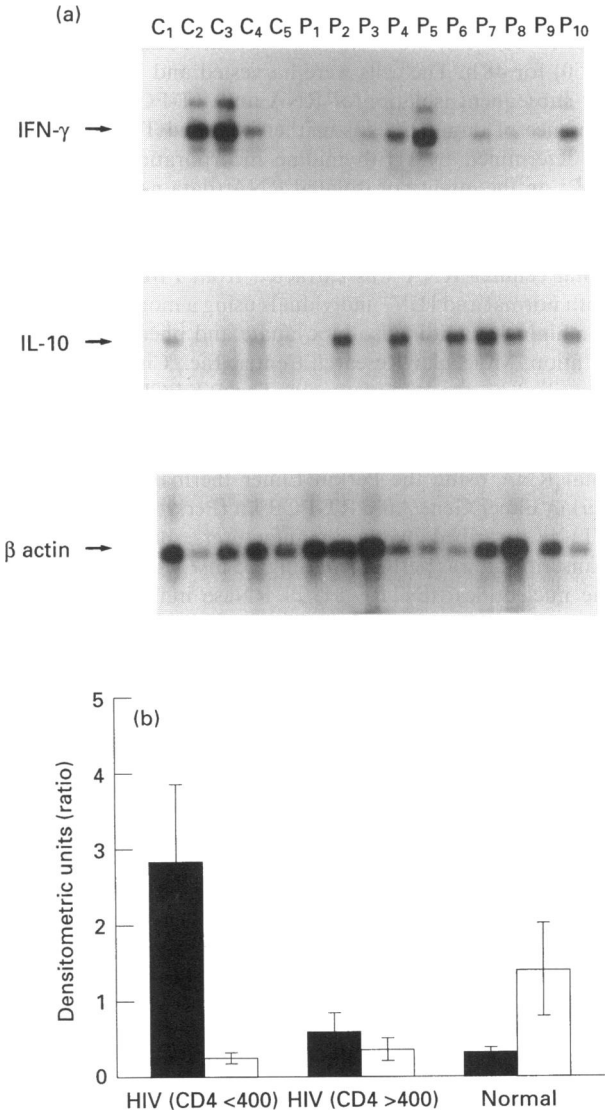

Fig. 2. (a) Expression of IFN-γ and IL-10 by PBL from a representative group of 10 HIV$^+$ irrespective of CD-4$^+$ T cell numbers and five normal individuals by semiquantitative RT-PCR analysis. Total RNA was reverse transcribed and equal amounts of cDNA were amplified by PCR using specific oligonucleotide primers as described in Materials and Methods. PCR amplified products were analysed by electrophoresis followed by hybridization using cDNA probes specific for IFN-γ, IL-10 and β-actin. C_1 to C_5 denote normal individuals. P_1 to P_{10} denote HIV$^+$ individuals. (b) Differential expression of IFN-γ (□) and IL-10 (■) in 30 HIV$^+$ and 16 normal individuals. IL-10, IFN-γ and β-actin PCR amplified bands as demonstrated by autoradiography were analysed by densitometry and assigned arbitrary units. For comparison, IL-10 and IFN-γ expression was normalized with respect to β-actin as a ratio of values for IL-10 and β-actin, and IFN-γ and β-actin respectively. Ratios are expressed as means of densitometric values ± s.e.

observed in 25 out of 30 patients and in the normal controls. IL-10 expression was observed to be more than 100-fold higher in 16 HIV$^+$ individuals and more than 10-fold higher in six HIV$^+$ patients with respect to IFN-γ expression. However, inverse correlation between IL-10 and IFN-γ was not observed in HIV$^+$ patients with advanced HIV disease characterized by

very low CD4 counts and a lower CD4/CD8 ratio. These results suggest that expression of IL-10 and IFN-γ in PBL is tightly and inversely regulated in normal individuals, and that in HIV infection, the level of expression of these two cytokines is reversed but continues to be inversely regulated.

Production of IL-10, IL-4 and IFN-γ in the culture supernatants of unstimulated PBL from HIV$^+$ individuals

To confirm that unstimulated PBL from HIV$^+$ individuals also produce relatively high amounts of IL-10 and consequently lower production of IFN-γ, we measured IL-10 (Table 1) by sandwich ELISA in the supernatants of PBL cultured for 24, 48 and 72 h without stimulation. IL-10 at levels of more than 16 pg/ml (detection limit of IL-10 ELISA) was produced by unstimulated PBL from a significantly higher number of HIV-infected as compared to normal individuals when the PBL were cultured either for 24 h (eight out of 11 HIV$^+$ *versus* two out of 10 normal individuals), 48 h (seven out of 11 HIV$^+$ *versus* three out of 12 normal individuals) or 72 h (43 out of 45 HIV$^+$ *versus* three out of 12 normal individuals). However, the levels of IL-10 produced by PBL from HIV$^+$ and normal individuals producing more than 16 pg/ml were not statistically significant (Table 1). These results indicate spontaneous production of IL-10 by PBL from HIV$^+$ individuals compared to HIV$^-$ normal controls. The levels of IL-4 and IFN-γ production in supernatants collected from unstimulated PBL from more than 80% of 28 HIV$^+$ individuals were found to be below the detection limits of ELISA (<16·0 pg/ml) (data not shown).

Production of IL-10, IL-4 and IFN-γ in the culture supernatants of mitogen-stimulated PBL from HIV$^+$ individuals

Enhanced expression of IL-10 and its inverse relationship with IFN-γ expression in unstimulated PBL from HIV$^+$ individuals may also be reflected in the PBL stimulated with mitogens. To determine the ability of PBL to secrete IL-10, IFN-γ and IL-4, the supernatants of PBL stimulated with PHA and anti-CD3 antibodies were harvested at 24, 48 and 72 h and analysed by ELISA. Two groups of HIV$^+$ individuals were observed based on the level of IL-10 production by PBL. Mitogen-stimulated PBL from one set of HIV$^+$ individuals produced low levels of IL-10 ($n = 25$) and were designated as low IL-10 producers based on an arbitrary value of <3 IL-10 stimulation index (SI). Mitogen-stimulated PBL from the second set of HIV$^+$ individuals ($n = 17$) produced high levels of IL-10 (IL-10 SI > 3) and were designated as IL-10 producers. The levels of IL-10 produced by this group of individuals were comparable to that produced by PBL from normal individuals (Table 2). However, IFN-γ production by PBL from HIV$^+$ individuals varied depending upon the time the supernatants were harvested. IFN-γ production by PHA-stimulated PBL was significantly lower at 48 h in HIV$^+$ individuals ($n = 15$) than in normal controls ($n = 16$). The levels of IFN-γ produced by PBL stimulated with PHA increased significantly after 72 h of stimulation in HIV$^+$ individuals ($n = 27$) compared to HIV$^-$ normal controls ($n = 31$, Table 3). Conversely, IFN-γ levels were not different ($P > 0{\cdot}05$) in anti-CD3 antibody-stimulated PBL of HIV$^+$ and normal controls (Table 3). These results suggest that the kinetics of IFN-γ production were slower in HIV$^+$ individuals and levels of IFN-γ by 72 h after stimulation with PHA were higher compared to normal controls.

IFN-γ is produced by CD4$^+$ T cells, CD8$^+$ T cells and natural killer (NK) cells. To determine the ability of CD4$^+$ T cells to secrete IFN-γ following stimulation, CD4$^+$ cells purified from both HIV$^+$ ($n = 6$) and normal individuals ($n = 8$) were stimulated with PHA for 48 h and analysed for IFN-γ expression by semiquantitative RT-PCR as described in Materials and Methods. There was no difference in the expression levels of IFN-γ in HIV$^+$ and normal individuals (Fig. 3).

To further determine the levels of Th2 type cytokines in HIV$^+$ individuals, PHA-stimulated PBL were analysed for IL-4 production in the supernatants harvested after 48 h of culture. The results show significantly lower levels of IL-4 in HIV$^+$ individuals with <400 CD4$^+$ T cells/mm^3 ($n = 15$) as compared to normal controls ($P < 0{\cdot}001$). In contrast, there was no difference in IL-4 production in HIV$^+$ individuals with >400

Table 1. Production of IL-10 by unstimulated PBL from HIV-infected and normal individuals

Subjects (n)	Day of supernatant collection	Number of individuals secreting IL-10*			P value†
		>16 pg/ml	(mean ± s.e.m.)	<16 pg/ml	
HIV$^+$ (11)	1	8	45·37 ± 15·07	3	<0·006
Normal (12)	1	2	35·0 ± 2·0	10	
HIV$^+$ (11)	2	7	50·5 ± 16·11	4	<0·05
Normal (12)	2	3	50·0 ± 11·9	9	
HIV$^+$ (45)	3	43	44·28 ± 14·16	2	<0·001
Normal (12)	3	3	37·5 ± 6·2	9	

PBL from HIV$^+$ individuals with less than 400 CD4$^+$ T cells/mm^3 and normal controls were cultured at a concentration of 2×10^6/ml for 24, 48 and 72 h without stimulation. Supernatants were harvested and assayed for IL-10 production by a sandwich ELISA.

* Means of only those samples which secreted more than 16 pg/ml of IL-10 were calculated.

† P value was calculated by χ^2 test which is a comparison between the number of HIV$^+$ and normal individuals secreting IL-10.

n, The number of samples examined.

Table 2. Production of IL-10 by PBL from HIV-infected and normal individuals following mitogenic stimulation

Subjects (*n*)	Stimulating agent	
	Anti-CD3 antibodies	PHA
	IL-10 (pg/ml ± s.e.m.)	
HIV$^+$		
Low IL-10 producers (25)	22.66 ± 6·06 (1)*	91·33 ± 22·69† (3·3)
IL-10 producers (17)	82·25 ± 24·72 (4)	412 ± 128·11 (20·5)
Normal		
IL-10 producers (16)	143·8 ± 34·63 (10·6)	550·25 ± 83·16 (40·4)

PBL (2×10^6/ml) from HIV$^+$ individuals and normal controls were stimulated either with anti-CD3 antibodies or PHA for 48 h. Supernatants were collected and assayed for IL-10 production by a sandwich ELISA. None of the normal subjects was a low IL-10 producer.

* Figures in parentheses indicate IL-10 stimulation index.

† Statistically significant ($P < 0·001$).

n, The number of samples examined.

CD4$^+$ T cells/mm^3 ($n = 5$) and normal individuals ($n = 10$) (Fig. 4).

DISCUSSION

The cellular basis for the gradual immunodeficiency in HIV infection is not well understood. The sequential loss of proliferative responses to recall antigens, alloantigens and lectins observed with disease progression may be attributed to perturbation of cytokine cross-regulation [3,12]. Various observations suggest that a selective dysfunction of T helper responses may occur with disease progression. We analysed the relative expression of cytokines produced preferentially by Th1 (IFN-γ) and Th2 type cells (IL-4 and IL-10) hypothesizing that HIV infection may preferentially induce expansion of CD4$^+$ T cells with Th2 cytokine secretion patterns. Unstimulated PBL from HIV$^+$ patients, particularly those with <400 CD4$^+$ T cells/mm^3, expressed significantly higher levels of IL-10 than did PBL from HIV$^-$ controls. In addition, IL-10 expression was inversely correlated with IFN-γ expression. However, mitogenic stimulation of PBL from HIV$^+$ individuals revealed decreased production of IL-4 and IL-10. Furthermore, the ability of total or positively selected CD4$^+$ PBL to produce IFN-γ was not impaired. These observations indicate dysregulation, but not preferential expression, of Th2 type cytokines in HIV infection.

Immunoregulatory cytokines play an important role in the regulation of HIV replication in CD4$^+$ T cells as well as in cells of the monocyte/macrophage system [23–25]. Detection of cytokines in the unstimulated PBL may thus indicate the level

Table 3. Production of IFN-γ by PBL from HIV-infected and normal individuals following mitogenic stimulation

Subjects (*n*)	Day of supernatant collection	Stimulating agent	
		Anti-CD3 antibodies	PHA
		IFN-γ (pg/ml ± s.e.m.)	
HIV$^+$ (15)	1	39·28 ± 6·6	37·5 ± 5·22
Normal (16)	1	42·6 ± 7·06	54·8 ± 7·82
HIV$^+$ (15)	2	48·25 ± 16·8	48·6 ± 3·6*
Normal (16)	2	41·4 ± 4·7	96·5 ± 20·6
HIV$^+$ (27)	3	81·4 ± 29·8	97·5 ± 20·1*
Normal (31)	3	55·7 ± 9·8	31·32 ± 7·74

PBL (2×10^6/ml) from HIV$^+$ individuals and normal controls were stimulated either with anti-CD3 antibodies or PHA for 24, 48 or 72 h. Supernatants were collected and assayed for IFN-γ production by a sandwich ELISA.

* Statistically significant ($P < 0·05$).

n, The number of samples examined.

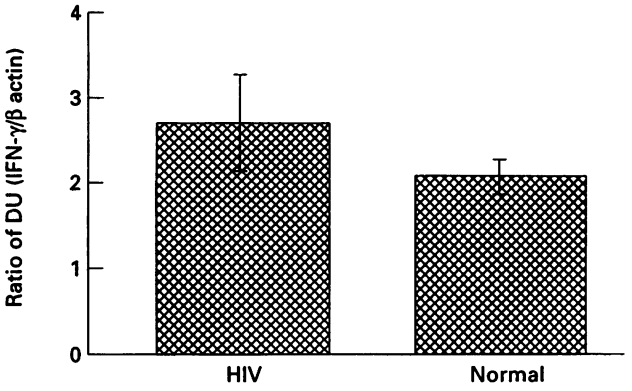

Fig. 3. IFN-γ expression by $CD4^+$ cells from PBL of six HIV^+ and eight normal individuals by semiquantitative RT-PCR analysis. Positively selected $CD4^+$ cells from PBL of HIV-infected individuals and normal controls were stimulated with PHA for 48 h. The cells were harvested and analysed for IFN-γ expression by semiquantitative RT-PCR as described in the legend to Fig. 2.

of HIV expression and disease progression [26]. HIV-inductive cytokines like TNF-α, IL-6 and GM-CSF trigger HIV replication [5,6] whereas transforming growth factor β, IFN-α and IFN-β have been shown to suppress viral replication [6,27–30]. However, IFN-γ exerts dichotomous effects on HIV replication depending on experimental conditions [6,28,29,31]. It directly enhanced viral replication in U1 promonocytic cell line but prevented replication following stimulation of cells with PMA [29].

Our results show that IFN-γ expression, as determined by RT-PCR analysis, is reduced in unstimulated PBL from HIV^+ patients with <400 $CD4^+$ T cells/mm^3. Low levels of IFN-γ expression are in concert with the high levels of IL-10 expressed in this group of patients. To our knowledge, this is the first report describing IL-10 upregulation and its inverse correlation with IFN-γ expression in unstimulated PBL of HIV^+ patients (Fig. 2a,b). This correlation was lost in patients with <50 $CD4^+$ T cells/mm^3. However, an inverse correlation in the production of IL-10 and IFN-γ as determined by ELISA could not be ascertained because

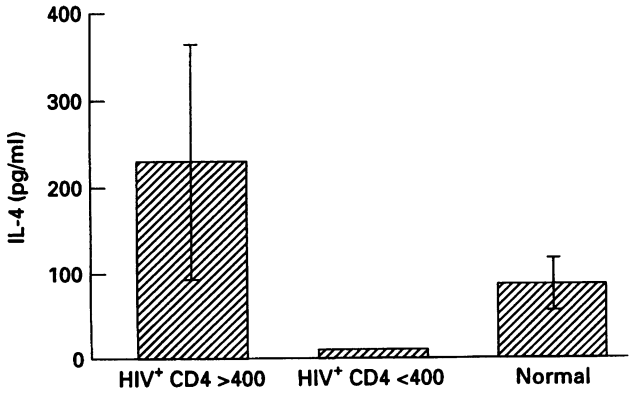

Fig. 4. IL-4 production by PBL of HIV-infected individuals and normal controls. PBL stimulated with PHA for 48 h were analysed for IL-4 production by ELISA as described in Materials and Methods.

IFN-γ produced by unstimulated PBL was below the sensitivity level of the assay.

The effect of HIV infection on IFN-γ production is not clear. Both low and high levels of IFN-γ production by PBL from HIV^+ individuals have been reported [12,14,32–34]. Our results revealed that IFN-γ production is a function of the activation signal (anti-CD3 antibodies *versus* PHA) and is time dependent. Anti-CD3 antibodies induced low levels of IFN-γ by PBL from HIV^+ individuals compared to controls. This is consistent with the observation indicating an impairment in the CD3 activation pathways in HIV^+ individuals [35]. However, stimulation of PBL with PHA revealed differences in the kinetics of IFN-γ production. Peak levels of IFN-γ were detected on day 2 by PBL from normal controls as compared to day 3 in PBL from HIV^+ individuals suggesting a slower rate of IFN-γ production by PBL from HIV^+ individuals. These results are in agreement with the high levels of IFN-γ detected in serum and in the supernatants of PBL of HIV^+ individuals [32–34]. It has been suggested that these high levels of IFN-γ are produced by $CD8^+$ T cells [14]. To determine the ability of $CD4^+$ cells to produce IFN-γ, we analysed IFN-γ production following stimulation of positively selected $CD4^+$ cells, which includes $CD4^+$ T cells and monocytes. IFN-γ production by $CD4^+$ cells of HIV^+ and normal individuals was comparable, which suggested that the ability to produce IFN-γ was not impaired in $CD4^+$ T cells of HIV^+ individuals. The biological significance of enhanced production of IFN-γ as compared to IL-2 is not clear, but it has been suggested that enhanced IFN-γ production may contribute to inhibition of virus replication and the increased susceptibility of T cells to apoptosis during HIV infection [36].

In this study, we observed spontaneous production of IL-10 by unstimulated PBL. IL-10 is produced by monocytes, EBV-transformed B cells and both Th1 and Th2 type T cells [7–11,37]. Among T cells, IL-10 is primarily produced by the Th2 subset [37]. IL-10 has been reported to be upregulated in B cell lymphomas from HIV^+ patients, but was attributed to EBV transformation [10,11]. The patients in the present study exhibited neither B cell lymphomas nor infectious mononucleosis. However, the possibility that IL-10 expression could be due to the presence of opportunistic infections cannot be ruled out. We previously found that patients with high levels of EBV replication had an increased risk of rapid HIV disease progression [38]. It is not known whether there is a direct correlation between high EBV load and IL-10 expression in HIV infection, but overexpression of IL-10 in this setting may contribute to the observed B cell hyperplasia and hypergammaglobulinaemia owing to its activity as a B cell growth factor [9,39]. Spontaneous production of IL-10 by unstimulated PBL from HIV^+ individuals may also contribute to the defective antigen/recall response in HIV infection due to its downregulatory effects on IFN-γ, IL-2 and IL-12 production, MHC II molecule and B7-1 expression and antigen presentation [7–9,40]. There is evidence to suggest loss of production of IL-2, a Th1 type cytokine, by PBL of HIV^+ individuals [1,2,12,14–18,24] which may contribute to the development of immune unresponsiveness. This may eventually result in the loss of innate immunity and cellular immune responses (CMIR). IL-10 has also been shown to inhibit HIV replication [41]. Spontaneous production of IL-10 in HIV^+ individuals

as reported by us and other investigators [14] raises the question of the effect of IL-10 on *in vivo* HIV replication and virus load.

Unstimulated PBL from HIV^+ individuals spontaneously secrete relatively high levels of IL-10, yet produce low levels of IL-10 following stimulation with PHA. Spontaneous production of IL-10 by PBL from HIV^+ individuals may be attributed to the action of HIV regulatory genes such as *tat* which has been shown to upregulate IL-10 production in T cell lines [42]. However, the mechanism for the loss of IL-10 production by PHA-stimulated PBL particularly from low IL-10 producers is not understood. Our results suggest that IL-10 is mainly produced by non-T non-B cells in the PBL of HIV-infected individuals [43] which may not be able to produce IL-10 following stimulation with PHA. Furthermore, IL-10 production is differentially regulated in normal T cells and monocytes: TNF-α regulates IL-10 production by monocytes [44] whereas IL-6 and IL-12 regulate IL-10 production by T cells (manuscript submitted). The impairment in IL-10 production may be due to the dysregulated production of IL-10 regulating cytokines in HIV infection. The significance of loss of IL-10 induction by mitogen-stimulated PBL from a subset of HIV^+ individuals is not known, but preliminary results suggest an association of IL-10 production with unresponsiveness to recall antigens [43]. Studies are in progress to investigate such an association, including the significance of IL-10 production with respect to clinical manifestations.

We have also observed significantly lower levels of IL-4 by PHA-stimulated PBL from HIV^+ individuals with <400 $CD4^+$ T cells/mm^3. Various laboratories have reported differing levels of IL-4 and IL-10 levels by mitogenically stimulated PBL from HIV^+ individuals. Our results disagree with earlier reports showing enhanced levels of IL-10 and IL-4 production by PBL following PHA stimulation [2,12]. However, lower levels of IL-4 and IL-10, agreeing with our results, have also been reported [14–18]. Variable results on cytokine production by different laboratories may be explained by different measurement protocols, stage of disease, the time of PBL collection, opportunistic infections and different antiviral medications.

Unfractionated PBL, which contain other cells in addition to $CD4^+$ Th1/Th2 cells, may not be the ideal source for analysis of Th1/Th2 cytokines. However, a cytokine profile of mitogen-stimulated PBL indicates the preferential activation of Th1/Th2 type cells that may be helpful in determining the prognosis of disease. A significant decrease of IL-4 and IL-10 in PBL of HIV^+ individuals with <400 $CD4^+$ T cell/mm^3 as observed in this study suggests downregulation of Th2 type cytokines. The question of Th1/Th2 type cytokines in HIV infections has recently been addressed [14–18]. Association of disease progression with a shift from Th1 to Th0 cells and preferential infection of Th2 type cells by HIV has been suggested [18]. It is possible that the loss of Th2 type cytokines may be due to the preferential killing of IL-4 producing Th2 type cells by HIV [17,18]. It is logical to study HIV-infected T helper cell cytokine profiles at the single cell level. Additional data are necessary to determine the extent of modulation of T helper cell pathways in HIV infections.

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