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STUDIES OF BLOOD MONOCYTES FROM PATIENTS INFECTED WITH THE HUMAN IMMUNODEFICIENCY VIRUS-1 (HIV-1)

By

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THESIS
Submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

In human immunodeficiency virus type 1 (HIV-1) infected patient's blood, $CD4^+$ T cells harbor HIV-1, but it is controversial whether $CD4^+$ blood monocytes carry the virus or not. Tissue macrophages are known to be infected. To determine if blood monocytes from HIV-1 seropositive patients contain HIV-1 antigen and genome, we separated monocytes and T cell subsets using monoclonal antibodies (mAbs) conjugated to magnetic beads and by monocyte adherence to glass. Monocytes were cultured with macrophage colony-stimulating factor (M-CSF or CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3). After 14 days in culture, cells were analyzed for the presence of HIV-1 antigen and multinucleated giant cells (MGCs). Freshly isolated cell subsets were analyzed for HIV-1 proviral DNA by polymerase chain reaction (PCR) using modified *env* (SK 68i/69i2) and *gag* (SK145i/150) primers. These primers are specific for HIV-1 *env* and *gag* sequences.

We found: (1) Monocytes cultured without depletion of $CD4^+$ T cells (11 of 11 patients) were HIV-1 antigen positive and showed dramatically increased spontaneous formation of MGCs. (2) Monocytes cultured after depletion of $CD4^+$ T cells (3 experiments) were HIV-1 antigen negative and MGC formation was markedly decreased. (3) In 14 subsequent patients analyzed by PCR, all patients were positive for HIV-1 proviral DNA in cells enriched for $CD4^+$ T cells. In 11 of 14 patients (79%), the monocyte fractions were HIV-1 proviral DNA negative, while in the remaining 3 patients, the monocytes were positive for HIV-1 proviral DNA.

In conclusion, the major reservoir for HIV-1 infection in human peripheral blood is $CD4^+$ T cells (14 of 14 cases). Fresh blood monocytes from HIV-1 seropositive patients were HIV-1 proviral DNA negative in 11 of 14 cases (79%). Blood monocyte-derived macrophages from HIV-1 seropositive patients may acquire HIV-1 infection in vitro from contaminating infected $CD4^+$ T cells. The pathogenic and clinical significance of HIV-1 infected monocytes (21% of patients) remains to be determined.

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This thesis is dedicated to my parents, my husband, Ren,
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LIST OF REAGENTS

FICOLL-HYPAQUE	Pharmacia
FBS	Fetal Bovine Serum (Hyclone)
IMDM	Iscove's Modified Dulbecco's Medium (Gibco)
PBS	Phosphate-buffered Saline, pH 7.4 (Gibco)
HBSS	Hanks' Balanced Salt Solution Modified (Flow)
BSA	Bovine Serum Albumin (Sigma)
GM-CSF	Granulocyte-Macrophage colony-stimulating factor (Genzyme)
M-CSF	Macrophage colony-stimulating factor (Genzyme)
IL-3	Interleukin-3 (Genzyme)
AMT	4'-Aminomethyl-4'-5'-8'-trimethylpsoralen hydrochloride (Lee Bio Molecular Research Lab)
DAB	3,3'-Diaminobenzidine tetrahydrochloride (Sigma)

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ARC	AIDS related complex
APC	Antigen presenting cell
ACD	Acid Citrate Dextrose
AZT	3'-Azido-2'3'-dideoxythymidine
BFU-E	Burst-forming unit, erythroid
CD	Cluster of Differentiation
CFU-blast	Colony-forming unit, blast
CFU-GEMM	Colony-forming unit, granulocyte-erythroid-macrophage-megakaryocyte
CFU-MEG	Colony-forming unit, megakaryocyte
CFU-S	Colony-forming unit, spleen
CR	Complement receptor
FcR	Fc receptor
FITC	Fluorescein isothiocyanate
HIV-1	Human immunodeficiency virus-1
HPP	High proliferative potential
IFA	Immunofluorescence assay
IL	Interleukin (e.g. IL-3,IL-4)
Ig	Immunoglobulin
k	Kilodalton
kb	Kilobase
LTR	Long terminal repeat
mAb	Monoclonal antibody
MGC	Multinucleated giant cell
MNC	Mononuclear cell

MHC	Major Histocompatibility Complex
m.w.	Molecular weight
MPC	Magnetic particle concentrator
ND	Not detected
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate-13-acetate
PE	Phycoerythrin
RT	Reverse transcriptase
TNF	Tumor Necrosis Factor
UV	Ultraviolet radiation

I. INTRODUCTION

I.1 Preamble

The human immunodeficiency virus type 1 (HIV-1) was identified as the etiologic agent of the acquired immunodeficiency syndrome (AIDS) in 1983 (1-4). Since then much has been learned about its structure, function and biology (5). The main targets of HIV-1 are the $CD4^+$ subset of T lymphocytes and mononuclear phagocytes. Although early studies of this virus focused on $CD4^+$ T lymphocytes as the main target cell (6-7), there is increasing evidence that infection of cells belonging to the mononuclear phagocyte system plays a crucial role in the pathogenesis and progression of this disease (8-12). Blood monocytes and various types of tissue macrophages have the CD4 receptor, thus, they are susceptible to HIV-1 infection. In the central nervous system, the monocyte-derived cells (microglia) appear to be the primary target for HIV-1 infection (13-16). These tissue macrophages are either mononucleated or multinucleated, and they actively synthesize viral RNA and produce virions.

The mononuclear phagocyte system, which includes blood monocytes and tissue macrophages, has important roles in immunological and inflammatory responses (17-18). Mononuclear phagocytes have surface receptors for cytokines that regulate their hematopoietic and immunologic functions. The hematopoietic growth factors, macrophage colony-stimulating factor (M-CSF or CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL-3), induce differentiation of this lineage into a monoblast, promonoblast, monocyte and tissue macrophage. The growth factors have been well studied and it has been shown that they can enhance viral replication in long-term cultures (19-21). However, there is controversy regarding whether or not peripheral blood monocytes are susceptible to HIV-1 infection. Many investigators have assumed that monocytes are HIV-1 infected (8-12). However

recent data from other laboratories (22-24) have indicated that blood monocytes from HIV-1 seropositive patients may not be infected in vivo.

Thus, there is a controversy regarding the question: are blood monocytes, from HIV-1 infected patients, infected with the virus?. The purpose of this thesis is to resolve this controversy. We plan to determine if, in HIV-1 infected patients, blood monocytes are actively infected with HIV-1 and/or carry HIV-1 proviral DNA.

In the following section, I will briefly review the structure, replication and pathogenesis of HIV-1, focusing mainly on the role of mononuclear phagocytes in this viral infection.

I.2 HISTORY

In 1981, a number of previously healthy homosexual adult men in Los Angeles, New York, and San Francisco were observed to have developed evidence of severe immunodeficiency (25-27). Some developed unusual opportunistic infections while others were found to have Kaposi's sarcoma. Initial immunologic evaluation of these patients showed a marked deficiency of cellular immune function and perhaps most striking, a selective loss of $CD4^+$ T cells (25-31). This condition, in its last stage, has been termed acquired immunodeficiency syndrome (AIDS). AIDS was subsequently found to be caused by a newly discovered pathogenic retrovirus that was originally designated human T lymphotropic virus (HTLV-III) (1-2), lymphadenopathy-associated virus (LAV) (3), or AIDS-associated retrovirus (ARV) (4), and now is called HIV-1 (32).

I.3 THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1): STRUCTURE AND REPLICATION

HIV-1 is a retrovirus of approximately 100 nm in diameter (5). By electron microscopy, virions have a characteristic dense, cylindrical protein core that encases the genomic RNA

molecules and viral enzymes. Surrounding the core is the membranous lipid envelope. In the virus core, along with the RNA that carries the virus' genetic information, is an enzyme known as reverse transcriptase (RT).

The genetic structure of the HIV-1 provirus consists of long terminal repeat (LTR) elements at each end, along with three structural genes that are essential for virus replication. The *gag* gene encodes a polyprotein precursor that is subsequently cleaved by the viral protease during maturation to produce the core protein; the *pol* gene encodes a precursor protein with the order NH_2-protease-reverse transcriptase-endonuclease-COOH which is cleaved to yield the three enzymes; the *env* gene encodes a heavily glycosylated polypeptide precursor (gp160) that is processed to form the exterior glycoprotein (gp120) and transmembrane glycoprotein (gp41). In addition to these genetic elements, HIV-1 contains at least six accessory genes: *tat, rev, nef, vif, vpr*, and *vpu* (33). The expression of these genes has an impact on the pathogenic mechanisms exerted by the virus. The *tat* gene plays an important role in both transcriptional and post-transcriptional events of virus replication, while *vpr* is only a weak transcriptional activator(34). *Rev* regulates the structural gene expression; *vif* promotes infectivity of cell-free virus; and *vpu* is required for efficient virion budding. The functions of *nef* is uncertain. It has also been shown that *tat* can specifically inhibit antigen-induced lymphocyte proliferation, which suggests that *tat* might directly contribute to the immunosuppression associated with HIV-1 infection (35).

The life cycle of HIV-1 begins with binding of the viral envelope to a cellular receptor. HIV-1 is the only retrovirus for which the nature of the cellular receptor (the CD4 antigen) has been defined (6-7). Thus, the distribution for the CD4 antigen in different cell types reflects the tropism of the virus, with the major target cells being the T-helper/inducer lymphocytes and the cells of the monocyte-macrophage lineage (6-12).

CD4 is a nonpolymorphic member of the immunoglobulin gene superfamily which is expressed on the surface of a functionally distinct population of T lymphocytes (36) and monocytes (37-38). It is a glycoprotein with a MW of 55k consisting of an extracellular region with four immunoglobulin-like domains, a transmembrane region and a cytoplasmic region (39).

The essential function of CD4 is to bind the nonpolymorphic region of major histocompatibility complex (MHC) class II molecules during antigen presentation. Although it has been recognized that CD4 is also present in monocytes, the frequency of $CD4^{+}$ monocytes has been unclear, ranging from 10 to 90% (40-41). Recently Filion et al have shown that all human monocytes are $CD4^{+}$ (42), but the antigenic density of surface CD4 is much lower than in T cells. In addition, it has been shown that monocytes contain intracellular CD4 (42-43). The function of intracellular CD4 is still unknown.

The cell-surface expression of CD4 is required not only for viral infection, but also for cell fusion, which leads to syncytia formation (44). Syncytia are giant, multinucleated structures that form when HIV-infected cells fuse with uninfected cells. They occur because viral envelope glycoprotein on the surface of infected cells binds to CD4 molecules on uninfected cells. After HIV-1 binds to the CD4 molecule, the virus is internalized and uncoated. The precise mechanism of virus entry into the target cells is unclear. CD4 receptor-mediated endocytosis appears to be the mode of free virus infection (45) in addition to CD4-dependent entry via direct fusion between the viral and plasma membranes (46). It is also possible that molecules other than CD4 are responsible for HIV-1 internalization. This is consistent with the observation that mouse cells expressing the human CD4 gene will bind virus but will not permit infection and will not fuse with other cells expressing the HIV-1 envelope protein (47).

It has been shown that the CD4 molecule is, in fact, the high-affinity receptor for the virus (48). The domains involved in this interaction have been mapped on both the envelope and the CD4 proteins (49-51). It appears that a region of the extracellular major envelope glycoprotein (gp120) forms a pocket that constitutes the CD4-binding domain. For the CD4 molecule, the binding site resides within an amino-proximal region comprising the first immunoglobulin-like domain, a site between amino acids 32 and 47 (49). The specific anti-CD4 monoclonal antibodies (mAbs) that bind to this region, such as OKT4A and Leu3a, can block the interaction between CD4 and gp120.

Once inside the cell, the genomic RNA is transcribed to DNA by the viral enzyme reverse transcriptase (52). The proviral DNA, which can exist in a linear or circular form, is integrated into the host chromosomal DNA in a process dependent on an endonuclease encoded by the viral *pol* gene (53). An unusual feature of HIV-1 infection compared to most other retroviruses is the accumulation of large amounts of unintegrated viral DNA in the infected cells (54). The expression of the HIV-1 gene is stimulated initially by the action of select inducible and constitutive host transcription factors with binding sites in the long terminal repeat, which then leads to the sequential production of various viral mRNAs. The first mRNAs produced correspond to the multiply spliced species of approximately 2.2 kb encoding the *Tat*, *Rev*, and *Nef* regulatory proteins. Subsequently, the viral structural proteins are produced, allowing the assembly and morphogenesis of virions. The free HIV-1 virions that are produced by viral budding from the host cell can then reinitiate the retroviral life cycle by infecting other $CD4^+$ target cells (54).

I.4 PATHOGENESIS OF HIV-1

The likely routes of transmission for HIV-1 have been established. Sexual transmission, transfusion of blood and blood products, contaminated needles shared by intravenous drug abusers, and perinatal transmission remain the principal routes of viral spread (55).

Very little is known concerning early events following HIV-1 infection in humans. The initial target cells in the rectum, genital tract or blood stream have not been identified, although cells of the monocyte-macrophage lineage and lymphocytes are good possibilities. Dissemination to target organs probably occurs through the blood, either in a cell-associated form or in plasma (56). It has been emphasized that not only whole blood but cell-free plasma and plasma-derived products from HIV-1 infected patients should be considered potentially infectious.

The major target organs for HIV-1 are the lymphoreticular, hematopoietic, and nervous systems. The critical cellular targets are $CD4^+$ T cells and mononuclear phagocytes. The $CD4^+$ T

cell is crucial in the generation of many immunologic functions such as (a) activation of macrophages, (b) induction of cytotoxic or suppressor T-cell functions, (c) secretion of growth or differentiation factors for lymphoid and hematopoietic cells, and (d) induction of B-cell function. Monocyte-macrophages are crucial in (a) phagocytosis and elimination of foreign organisms, (b) presentation of antigen to lymphocytes, and (c) secretion of regulatory monokines. Hence, infection and virus-induced dysfunction of these cell types would be expected to result in immunologic abnormalities. However, examination of cells from lymph nodes and peripheral blood from patients with AIDS and AIDS-related complex (ARC) has revealed a very low frequency of viral RNA synthesis, generally occurring in 1/100,000 to 1/10,000 total mononuclear cells (57). It is possible that a large proportion of cells may be latently infected (containing integrated proviral DNA but not expressing viral mRNA or protein). The development of gene amplification (polymerase chain reaction) methodology has made it possible to detect HIV-1 infected cells that are not expressing virus. By the PCR technique, it has been confirmed that the $CD4^+$ T cells are the reservoir for HIV-1 in the peripheral blood of infected individuals, and the frequency of $CD4^+$ T cells that contain HIV-1 proviral DNA is at least 1/100 cells in patients with AIDS according to most recent studies (22).

I.5 HIV-1 INFECTION OF HUMAN MONONUCLEAR PHAGOCYTES

It has been shown that normal, blood-derived monocytes in culture can be infected in vitro with HIV-1 (8-10), and virus can be cultured from peripheral blood monocytes (11-12). However, virus production by monocytes is not detectable in the majority of patients, does not correlate with disease severity, and requires amplification with lymphoblast coculture (58). There is evidence that HIV-1 infection in mononuclear phagocytes can be mediated by antibody-dependent enhancement via Fc receptor (FcR) or by complement receptors (59-60) which still requires CD4 interaction with the virus glycoprotein (61). The process of viral entry is also the result of complex interactions between host and viral factors in addition to CD4 and gp120

molecules. It has been observed that when normal T cells were infected in vitro, viral replication was detected within 3-6 days by immunofluorescence assay (IFA) and RT activity in culture supernatant. However, when normal monocytes/macrophages were infected in vitro, viral replication was not detected until after 7-10 days in culture (8, 62). Kim et al (62) have shown that this difference in virus production is not due to differences in the mechanism of viral replication or the amount of CD4 receptor on the cell surface. They concluded that in the monocytic cell line U937, there is a major limiting event during viral entry into the cell. This limiting event may also apply to the monocytes/macrophages. One possible explanation for this is that factors other than CD4 are involved in viral entry. The other possibility is that the CD4 molecule of monocytes may differ from that of T cells, perhaps by post-translational modification. Kim et al (62) also demonstrated that once the virus entered the host cell, reverse transcription, use of the long terminal repeat, RNA expression, and production of virus particles was about as efficient in monocytes as in T cells.

In vitro, the fate of HIV-1 virions in mononuclear phagocytes is quite different from those in T cells . Ultrastructural analysis of HIV-infected macrophages two to six weeks after infection (time intervals where 60-90% of cells express both HIV-specific mRNA and proteins) shows little or no virions at the plasma membrane. Yet these infected cells contain large numbers of viral particles. Virus is localized almost exclusively to intracellular vesicles. HIV-1 not only accumulates within these intracellular vesicles but also assembles and buds from the vesicle membranes (19). In contrast, HIV-infected T cells show hundreds of viral particles associated with the plasma membrane and there is no intracellular accumulation of mature, or even immature, virions. Most importantly, these in vitro observations have been confirmed in AIDS patients. Macrophages in the brain of an infected individual also show intracellular localization of viral particles within vesicles and little or no virus was detected at the plasma membrane (13). Such viruses, sequestered and protected from host immunity within cytoplasmic vesicles, may represent a reservoir for continued infection. Release of infectious viruses from this macrophage

reservoir and dissemination of HIV-1 into other macrophages or T cells could be initiated by any agent that perturbs macrophage function.

It is known that immunological activation is required for replication of HIV-1 in peripheral blood T lymphocytes in vitro, at least partly as a result of an increase in the effective concentrations of cellular transcription factors required for HIV-1 expression (63). The HIV-1 LTR contains a number of cis-acting sequences that are targets for transactivators including the cellular DNA-binding protein NF-kB. NF-kB is a pleiotropic mediator of gene regulation involved in the transcriptional activation of several immunoregulatory genes, cytokine genes, and viral promoters (64). Activation of the HIV-1 LTR in proliferating T cells after treatment with mitogens or phorbol esters is associated with synthesis of NF-kB (65). It was suggested that in T cells, HIV-1 gene activity and multiplication were dependent on the induction of NF-kB. Although NF-kB binding activity was inducible by TNF-α and phorbol-12-myristate-13-acetate (PMA) in monocytic cells, it was not required for HIV-1 LTR activation or HIV-1 replication (65). In vivo, it is likely that HIV-infected mononuclear phagocytes may be primed in some manner by HIV-1 infection such that subsequent antigenic stimulation of these cells leads to the production of TNF-α and other cytokines. Continued secretion of TNF-α from activated, HIV-1 infected mononuclear phagocytes may contribute to viral pathogenesis by increasing the lytic spread of HIV-1 through a T cell population.

PCR analysis of highly-enriched, sorted monocytes from HIV-1-infected individuals (22-24) has indicated that only a minority of such individuals have demonstrable HIV-1 DNA in their peripheral monocytes, whereas all have HIV-1 DNA in their $CD4^+$ T cells. By using the same method, it was suggested that bone-marrow myeloid stem cells are also not susceptible to HIV-1 infection (66-67). However, in certain tissues (central nervous system, lymph nodes, lung) the frequency of productively infected cells may exceed 1 in 10 (68). In each of these tissues, the infected cells are not $CD4^+$ T cells; they are macrophages (13-16, 69-71). Multinucleated giant cells have also been observed in macrophage cultures infected in vitro (9), and were the major cell type synthesizing HIV-1 in the brain of infected individuals (13). However, detailed analysis

of the extent of macrophage involvement in HIV-1 disease is still incomplete. It seems that, unlike the $CD4^+$ T cells and tissue macrophages, the relatively immature monocytes found in the peripheral blood are less susceptible to infection by HIV-1. It appears that the capacity to obtain productive infection of the mononuclear phagocytes may be dependent upon cellular factors that change with differentiation.

Macrophages also release many secretory products that have direct effects on nerve growth, function and repair of injury. Inappropriate secretion of these monokines (IL-1, IL-6, TNF-α, M-CSF etc.) by HIV-infected macrophages in brain may induce both neurological symptoms and tissue injury. Recent reports suggest induction of prostaglandins, IL-1, TNF-α and IL-6 in macrophages by gp120 (72-74).

It has been shown that hematopoietic growth factors can enhance monocyte survival and differentiation into macrophage (75). Recent data demonstrated that they can also alter HIV-1 production by mononuclear phagocytes infected in vitro (19-21). But the mechanism of their effect on HIV-1 replication has not been fully characterized. It is of note that M-CSF has been shown by Gendelman et al (19) to enhance HIV-1 production by macrophages. In the case of M-CSF-stimulated macrophages, most of the HIV-1 production seemed to be sequestered in vacuoles; only low level virion production was detected in the supernatant by RT activity, while with GM-CSF, abundant virus was released into the medium (20). GM-CSF also markedly enhances the anti-HIV-1 effect of AZT and closely-related drugs in macrophages. IL-3 enhances HIV-1 production as revealed by detection of p24 antigen in the supernatant from macrophage cultures infected in vitro (21).

I.6 GENERAL ASPECTS AND DEVELOPMENT OF THE MONONUCLEAR PHAGOCYTIC SYSTEM

The mononuclear phagocyte system is composed of their bone marrow precursors, blood monocytes, tissue macrophages, as well as several other highly specialized macrophages (17).

The precursors of monocytes/macrophages develop from pluripotential stem cells that become committed to this lineage as they divide and differentiate. Glycoprotein hormones termed colony-stimulating factors (CSF), especially the macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and interleukin-3 (IL-3) or multipotential CSF induce differentiation of this cell lineage into a monoblast, promonocyte, monocyte and eventually tisssue macrophage (76-78). The earliest monocytic precursors in the marrow include monoblasts and promonocytes. They mature within the bone marrow to monocytes and then enter the blood. After circulating for about 1-3 days, monocytes leave the blood to enter the tissues, where they differentiate into macrophages and/or specialized cells.

In Wright-Giemsa stained preparations, the human blood monocyte appears as a large (10-18μm diameter) cell with a horseshoe-shaped nucleus and grayish-blue cytoplasm that frequently contains faint azurophilic granules. Ultrastructurally, the monocyte possesses ruffled membranes, a well-developed Golgi complex and many intracytoplasmic lysosomes.

The mononuclear phagocytes have diverse surface receptors that allow them to interact with a range of hormones, proteins, polysaccharides, and lipids (79). Mononuclear phagocytes have receptors that bind either the Fc fragment of IgG (FcR) or to the complement proteins (CR). The function of both is to promote the uptake of antibodies- and/or complement-coated molecules. Both receptors can act in synergy to increase the rate of uptake of an opsonized particle.

With maturation of the monocyte to the macrophage, the cell greatly enlarges and displays a cytoplasm containing abundant granules. They are long-lived cells with significant rough-surfaced endoplasmic reticulum and mitochondria. As the cell matures from monocyte to macrophage, its ability to phagocytose particles increases and requires a large number of lysosomes. Maturation of peripheral blood monocytes in vitro is associated with a decrease in membrane CD4, while HLA-DR and FcR III expression increase along with lysozyme secretion (80).

Macrophages may also undergo fusion into large multinucleated giant cells (MGCs), which are found in a variety of granulomatous inflammatory diseases formed in response to foreign bodies, viruses, and bacteria (81-82). It has been reported that interleukin-4 (IL-4) induces MGC formation by blood monocytes in culture (83).

A fundamental role for macrophages in the steady state and during disease is regulation of tissue function. This regulatory role is mediated by the many secretory molecules released by the macrophage under a variety of pathophysiological conditions. Among the key biological molecules released by macrophages are those that participate in host defence and include IL-1, IL-6, TNF-α, and interferons α and ß. All these are secreted upon uptake of microbial products or particles opsonized by complement component C3 and Ig.

The tissue macrophages are divided into two different types: 1. the phagocytic macrophage whose predominant role is to remove particulate antigens, such as lung alveolar macrophages and liver Kupffer cells; 2. antigen-presenting cells (APCs) which are found especially in lymphoid tissues (interdigitating follicular cells) and in the skin (Langerhans' cells). The function of APC is to take up and present antigen to lymphoid cells in association with the organism's own MHC (18).

I.7 RECOMBINANT GROWTH FACTORS

The GM-CSF, M-CSF and IL-3 genes have been cloned and cloned genes have been used to produce and purify isolate recombinant products. The cell sources, biological functions and chromosome location of the genes for these growth factors are shown in Tables 1,2,3 (76-77, 84-85):

i. Macrophage Colony-Stimulating Factor (M-CSF).

M-CSF or CSF-1, the macrophage-specific CSF, binds primarily to mononuclear phagocytes via a specific receptor (CSF-1 receptor). This CSF receptor is a tyrosine kinase identical to the c-fms proto-oncogene product. The CSF-1 receptor, of MW about 165k, has been

identified in monocytes and macrophages. The number of receptors increases with the state of macrophage maturation. M-CSF is a heavily glycosylated homodimer of MW 45k, and the M-CSF encodes a 26k protein. The M-CSF not only promotes the growth but also the differentiation of progenitor cells into macrophages and the survival of macrophages in culture. M-CSF is present in blood in physiological concentrations and may well be the key physiological regulator of blood monocyte levels. The half-life of M-CSF is about 10 minutes and its level is controlled by the number of macrophages, particularly in the liver and spleen.

ii. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF).

GM-CSF is a glycoprotein of MW 23k, and its GM-CSF gene encodes a 13.5k protein. The mRNA of GM-CSF is 1.0 kb in length. GM-CSF stimulates the proliferation of a progressively broader spectrum of progenitor cells: CFU-blast, CFU-GEMM, BFU-E, and CFU-MEG. It particularly induces differentiation to macrophages or granulocytes. GM-CSF is not normally detected in serum, and is probably released locally, within the marrow cavity and at peripheral sites of inflammation where its action is regulated.

iii. Interleukin-3 (IL-3).

IL-3 is a glycoprotein of 28k and its gene encodes a 15k protein. The IL-3 mRNA is 1.0 kb in length. The receptor for IL-3 has been identified on the common precursor to myeloid cells. IL-3 has reactivity over a broad range of hematopoietic progenitor cells. This factor supports the growth of multipotent progenitors as well as progenitors with a more restricted commitment status. The target cells of IL-3 also include granulocytes, macrophages, eosinophils, megakaryocytes, and mast cells. Because of its ability to stimulate the growth of the earliest detectable multipotential colony-forming cells in vitro, this growth factor has also been termed multi-CSF.

TABLE 1

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Gene location (human) and main cellular production source

Chromosome 5; T-lymphocytes, Endothelial cells, Fibroblasts.

Major biologic actions

1. **Stimulates formation of granulocyte and macrophage colonies in vitro.**
2. **Acts synergistically with other factors to stimulate megakaryocyte, last cell, and BFU-E colonies *in vitro*.**
3. **Enhances cytotoxic and phagocytic activities of mature neutrophils.**
4. **Increases cell-cell adhesion and oxidative metabolism of mature neutrophils.**
5. **Inhibits motility of mature neutrophils.**
6. **Enhances cytotoxicity and leukotriene synthesis of mature eosinophils.**
7. **Stimulates *in vivo* production of granulocytes, macrophages, and possibly platelets in mice, primates, and humans.**

TABLE 2

Colony-stimulating factor-1
(CSF-1, M-CSF)

Gene location (human) and main cellular production source

Chromosome 5; Macrophages, Endothelial cells, Fibroblasts.

Major biologic actions

1. **Stimulates formation of macrophage colonies in vitro.**
2. **Acts synergistically with other growth factors in colony formation *in vitro*.**
3. **Supports survival of differentiated macrophages *in vitro*.**
4. **Increases antitumor activity of macrophages and their secretion of O_2 reduction products and plasminogen activation.**

TABLE 3

Interleukin-3
(IL-3, Multi-CSF)

Gene location (human) and main cellular production source

Chromosome 5, T- lymphocytes.

Major biologic actions

1. **Stimulates formation of granulocyte, macrophage, eosinophil, and mast cell colonies *in vitro*.**
2. **Acts synergistically with erythropoietin to stimulate formation of BFU-E colonies and with CSF to stimulate HPP colonies.**
3. **Induces CFU-S and leukemic blasts into cell cycle.**
4. **Stimulates alone or synergistically with other growth factors the *in vivo* production of all myeloid cells in mice and primates.**

II. OBJECTIVES

Although cells of the mononuclear phagocyte lineage are well-known targets of HIV-1 infection, our review of the literature strongly indicates that there is variability in the facility with which various members of the cell lineage can be infected. It appears that the more mature the cell, the easier it is to infect in vitro, and immature cells are not susceptible to HIV-1 infection. For example, although a previous report indicated that bone-marrow stem cells may be infected in AIDS (86), and that isolated myeloid progenitor stem cells ($CD34^+$) are susceptible to infection (87), data from other investigators (66-67) indicates that $CD34^+$ myeloid stem cells are not susceptible to HIV-1 infection. Likewise, because blood monocytes are CD4 positive, it is widely assumed that they are infected by the HIV-1. Several reports appear to confirm such a presumption and there are methods designed to allow the isolation of HIV-1 from blood monocytes (11-12). It is supposed that such monocytes carry monocytotropic virus as opposed to the lymphocytotropic virus carried by T cells. The concept of cellular tropism due to viral properties has been controversial. Recent reports have suggested that contrary to what has been reported, blood monocytes may not be infected with HIV-1 (22-24).

In order to determine if blood monocytes from HIV-1 seropositive patients are infected with HIV-1, I planned several experiments. First, I wanted to know if fresh blood monocytes express viral antigen. Second, I set up monocyte cultures isolated by standard-glass-adherent techniques to look for expression of HIV-1 antigen and multinucleated giant cell (MGC) formation. Third, I asked what the effect of depletion of $CD4^+$ T cells was on the expression of HIV-1 antigen and MGC formation by monocytes cultured from patients with HIV-1 infection. Finally, I asked if monocytes carry HIV-1 viral DNA or not.

In summary, the objectives were as follows:

1. To study the effect of GM-CSF, M-CSF and IL-3 on the expression of HIV-1 antigen and MGC formation by blood monocytes cultured from HIV-1 infected patients.

2. To study the effect of depletion of $CD4^+$ T cells on HIV-1 antigen expression and MGC formation by blood monocytes cultured from HIV-1 infected patients.

3. To perform PCR analysis to detect the presence of HIV-1 proviral DNA in purified fresh monocytes as well as in $CD4^+$ T cells and $CD8^+$ T cells from HIV-1 infected patients.

III. MATERIALS AND METHODS

III.1 BLOOD DONORS

Heparin-(50 units heparin/ml of blood) and citrate-(ACD, from Becton-Dickinson) containing tubes were used to collect 5 to 20 ml of venous blood from asymptomatic HIV-1 positive patients and hemophilic patients attending the comprehensive AIDS care clinic at the Ottawa General Hospital. Informed consent was obtained from each patient. Blood from normal donors was also collected as described, as well as from the Canadian Red Cross (Ottawa).

III.2 ISOLATION OF MONONUCLEAR CELLS (MNCs)

20 ml of peripheral blood was diluted to 35 ml with PBS and layered over 12.5 ml Ficoll-Hypaque discontinuous gradient. After centrifugation at 1800 rpm for 30 minutes (with brake off), the MNCs were isolated and collected from the interface. The cells were washed twice with PBS at 1000 rpm for 10 minutes and resuspended at $3x10^6$ cells/ml in IMDM-20% FBS.

III.3 DIRECT ENRICHMENT AND CULTURE OF ADHERENT MONOCYTES IN 8-WELL SLIDE CHAMBERS

1. MNCs were added to 8-well slide chambers (Lab-Tek, Gibco, 0.3ml/well) and incubated for 1 hour at 37°C in a 5% carbon dioxide (CO_2) atmosphere. After incubation, the nonadherent cells were removed and the wells were rinsed 4 times with IMDM. At this point, some slide chambers were dismantled and the slides then dried for studying fresh monocytes, while 0.3 ml of growth medium (IMDM-10%FBS) containing growth factors (50U/ml) were added to others. Slide chambers were placed on a humidified Petri-dish (Becton-Dickinson), and incubated for 7 or 14 days at 37°C in 5% CO_2.

2. For evaluation of the effect of growth factors on monocyte culture, we set up 2 slides as follows:

Slide1:

C	GM	M	IL-3
C	GM	M	IL-3

Slide 2:

C	GM	M	IL-3
C	GM	M	IL-3

C = Control GM = GM-CSF

M = M-CSF IL3 = Interleukin 3

3. On day 7, a set of cultures were terminated and processed for staining as described below. Fresh growth factors (50U/ml) were added to the day 14 set of cultures (0.15 ml removed, 0.15 ml fresh medium added).

4. On day 7 or day 14, HIV-1 virions were inactivated with AMT (4'-Aminomethyl-4-5'-8-trimethylpsoralen hydrochloride) and long-wave ultraviolet (UV) light (88). Exposure of HIV-1-infected cells to 5 µg /ml of AMT was followed by 5 minutes UV irradiation (320-380 nm). Then the cells were carefully washed as follows: (a) rinsed once with HBSS, (b) rinsed twice with 1% BSA in HBSS, (c) rinsed once with PBS. The cells were incubated with the buffer for 5 minutes before changing the buffer.

5. The slide chambers were dismantled and the slides then dried for at least 2 hours or overnight in a vacuum chamber. After drying, the slides were wrapped with Saran-Wrap and stored at -20°C.

6. The slides were stained using the immunoperoxidase assay to characterize HIV-1 antigen expression.

III.4 SUMMARY OF THE PROCEDURE FOR IMMUNOPEROXIDASE STAINING FOR HIV-1 ANTIGEN

1. Fixation: Slides were removed from the freezer and allowed to warm up to room temperature before fixation for 10 minutes with a fixative containing paraformaldehyde (2%), L-lysine (0.1M) and sodium periodate (0.01M); this step maintained optimal cellular morphology. For intracellular antigens, the cells were permeabilized by fixation with cold acetone (4°C) for 5 minutes.

2. All subsequent steps were done at room temperature. The slides were incubated in humid chambers (Petri dishes containing wet filter paper and wood sticks to support the slides); for washing, the slides were transferred to Coplin jars containing PBS, incubated for 5 minutes and the procedure repeated 3 times.

3. Blocking of non-specific binding sites was done with a blocking buffer that included Bovine Serum Albumin (BSA, 1%) and normal goat serum (5%, Gibco).

4. Blocking of endogenous myeloperoxidase and eosinophil peroxidase. The blocking buffer included sodium azide (0.1%) and glucose oxidase (1U/ml) /glucose (10mM) (89). The glucose oxidase/glucose reaction releases nascent hydrogen peroxide (H_2O_2) which efficiently inhibits peroxidase in the presence of sodium azide.

5. Antibody (polyclonal IgG fraction) from asymptomatic HIV-1 donors (AIDS Research and Reference Reagent Program, NIH, USA) was biotinylated in our laboratory using a Biotinylation Kit (Amersham). The antibody had been previously titrated, and was diluted in 1% BSA in PBS. Control staining included omission of the anti-HIV-1 antibody (replaced with 1% BSA in PBS).

6. Streptavidin-Peroxidase (from Jackson Immunoresearch Laboratories Inc.) was diluted in 1% BSA in PBS to its optimal dilution and added to slides. After 30 minutes incubation, the slides were washed.

7. Peroxidase Substrate solution was prepared immediately before use. It contained 3,3'-Diaminobenzidine Tetrahydrochloride (DAB) (0.05%, Sigma), H_2O_2 (0.05%, Fisher) and sodium azide (0.3%, Fisher). The latter two reagents were used again as a monocyte myeloperoxidase inhibitor (90). The reaction was carried out for 5 to 10 minutes and stopped by washing the slides in water. Then the slides were air-dried.

8. Counter-staining with Gill-3 hematoxylin (Fisher) involved flooding slides with hemotoxylin for 60 seconds, then rinsing in distilled water for 30 seconds. After air drying, the slides were mounted with DAKO glycergel (Dimension Laboratories Inc.).

9. The same procedures were applied to stain for cell markers using mAbs.

III.5 WRIGHT-GIEMSA STAIN

The slide was flooded with 1-2 ml of Wright-Giemsa Stain (Sigma). After 1 minute, an equal volume of deionized water (pH 6.8-7.2) was added for 1-3 minutes, then the slide was rinsed briefly in deionized water and air-dried. After air-drying, the slide was mounted with Permount (Fisher).

III.6 CELL SUBSET ISOLATION USING MAGNETIC BEADS

1. Blood samples were collected in citrate-containing-tubes (ACD, from Becton-Dickinson) and cooled down to 2-4°C by incubation on ice. 4 ml of whole blood was aliquoted to 2 polypropylene tubes (2 ml/tube) and samples were kept on ice.

2. The magnetic beads (Dynal) necessary for isolating the $CD4^+$ T cells, $CD8^+$ T cells, $CD2^+$ T cells and $CD19^+$ B cells were:

CD4 M-450 Dynabeads: 250 µl/ml.

CD8 M-450 Dynabeads: 300 µl/ml.

CD2 M-450 Dynabeads: 30 µl/ml.

CD19 M-450 Dynabeads: 12.5 μl/ml.

The vial containing M-450 Dynabeads was mixed well, and an appropriate volume to obtain a 10-20 : 1 ratio of beads to cells was added to the polypropylene tube. The tube was placed on a magnetic particle concentrator (MPC) for 60 seconds, then the fluid was removed by pipette. The tube was removed from the MPC and 5 ml of 1% FBS in PBS was added. The same procedure was repeated and the washed Dynabeads M-450 were resuspended in the volume of 1% FBS in PBS originally pipetted from the vial.

3. Depletion/enrichment of lymphocytes was done by adding CD4 M-450 Dynabeads to the whole blood and incubating for 1/2-1 hour with gentle rotation at 4°C. After incubation, the tubes were put onto the MPC for 2-3 minutes, then the $CD4^+$ T cell-depleted whole blood was collected. This isolating procedure was then repeated for each cell subset using the appropriate Dynabeads. The isolated $CD4^+$ T cells and $CD8^+$ T cells were washed 3 times with 1% FBS in PBS. The PCR lysis buffer (1 x cell lysis buffer: 50 mM KCl, 10 mM Tris-HCl (pH8.3), 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% NP-40) and the required volume of proteinase K (Sigma) was mixed (6 μl of 10 mg/ml proteinase K per 1 ml of lysis buffer), and washed cells were resuspended in 100 μl lysis buffer in 0.5 ml microcentrifuge tubes ($6x10^6$ cells/ml). The monocyte fractions were isolated from CD2,4,8,19 depleted whole blood by density gradient centrifugation on Ficoll-Hypaque, washed 2 times with 1 x PBS, and the cells again resuspended in 100 μl lysis buffer in 0.5 ml microcentrifuge tubes ($6x10^6$ cells/ml). Samples were digested with proteinase K for 1 hour at 56°C, followed by heat inactivation at 95°C for 10 minutes on a DNA Thermal Cycler (Perkin Elmer, Cetus). The Dynabeads were removed from $CD4^+$ T cell lysate and $CD8^+$ T cell lysate by placing the tubes onto the MPC for 60 seconds, and transferring the supernatant to new 0.5 ml microcentrifuge tubes. DNA from $CD4^+$ T cells, $CD8^+$ T cells and monocytes was then ready for PCR analysis (stored frozen at -20°C).

4. Staining of fresh blood cell subsets was followed by lysis of erythrocytes. The purity of enriched monocyte, $CD4^+$ T cell, and $CD8^+$ T cell fractions was analyzed by immunofluorescence with anti-human leukocyte mouse mAbs and a flow cytometer (FACScan,

Becton Dickinson). The Coulter Immunoprep Epics Leukocyte Preparation System was also employed, which allowed leukocyte analysis after erythrocyte lysis. The procedure was as follows: 100 μl of whole blood before and after each depletion was pipetted into a 12x75 mm test tube, then 10 μl of mAbs (Becton Dickinson) was added as determined from previous titration studies [anti-Leu-3a (CD4) phycoerythrin (PE) conjugate, anti-Leu-2a (CD8) fluorescein isothiocyanate (FITC) conjugate, mouse IgG1-FITC conjugate (control), and IgG1 PE conjugate (control) (Table 4)], and samples were incubated for 15 minutes at room temperature. The test tubes were placed in the instrument and the 35 seconds CYCLE button pressed. The Q-Prep is a matched three-component reagent system which automatically adds predetermined amounts of reagent. ImmunoPrep A is an erythrocyte lytic agent; ImmunoPrep B is a leukocyte stabilizer, and ImmunoPrep C is a cell membrane fixative. Cells were then washed with PBS (pH 7.4) twice, resuspended in 1 ml of 1% paraformaldehyde and samples refrigerated in the dark until analysis. Refrigerated samples were analyzed within 24 hours.

5. Analysis of fluorescence intensity on stained blood cell subsets was performed using a FACScan flow cytometer. Daily calibrations and standardizations were performed to obtain comparable results using standard fluorescent beads. By utilizing the 90-degree-angle and forward-angle light-scattering properties of the cells, it was possible to identify and separately analyze either lymphocytes or monocytes as distinct from each other or from granulocytes by their size and granulation (light-scatter properties). A gate was drawn around the population of interest for further analysis. The data were analyzed as logarithmic fluorescence histograms of gated cells. Mean fluorescence intensity was used as a measure of the amount of binding of the antibodies.

TABLE 4

Monoclonal antibodies to human leukocytes

CD*	MW**	ANTIBODY	PREDOMINANT REACTIVITY	NORMAL RANGE
CD2	45-50k	Leu5a	E rosette receptor	78-88%
CD3	22-28k	Leu4	T cells(T cell receptor complex)	68-82%
CD4	55k	Leu3a	Helper/Inducer T cells, Monocytes, macrophages	35-55%
CD8	32,43k	Leu2a	Cytotoxic/Suppressor T cells, NK cells.	20-36%
CD14	80k	LeuM3	Monocytes, macrophages granulocytes	85% of Monocytes
CD19	95k	Leu12	B cells	5-15%

*CD = Cluster of Differentiation. This is the nomenclature adopted by the International Workshop on Human Leukocyte Differentiation Antigens (91)

**MW = Molecular weight of antigen (kilodaltons)

III.7 SUMMARY OF THE PROCEDURE FOR HIV-1 DNA AMPLIFICATION AND DETECTION OF AMPLIFIED PRODUCTS

The PCR analysis was done on 25 µl of cell lysate, equivalent to 1.5 x 10^5 cells, for each primer set. The PCR reaction was performed for 30 cycles on an automated DNA thermal cycler (Perkin Elmer, Cetus) in a final volume of 50 µl (92-93). The reaction mix contained 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.12% Tween-20, 0.12% NP-40, 0.2 mM each of the four deoxyribonucleotide triphosphates (dNTP), 25 pmoles of each primer, 1 Unit of thermostable DNA polymerase from Thermus aquaticus (Taq; Perkin-Elmer Cetus).

Each sample was initially amplified with primers (GH26 and GH27; Synthetic Genetics, San Diego, CA) to a conserved region of the human HLA-DQα locus to determine whether the preparation was suitable for PCR analysis. The thermoprofile consisted of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds for a total of 30 cycles. 10 µl of the amplified product was then denatured (95°C, 5 minutes) and hybridized (15 minutes, 56°C) with 250,000 cpm of ^{32}P-end-labelled GH64 probe in 150 mM NaCl. One-third of each reaction was separated by 10% polyacrylamide gel electrophoresis and positive hybridization product was identified by exposure to X-Omat AR film (Eastman Kodak Co., Rochester, NY) for 4-18 hours at -70°C. Generation of an amplified HLA-DQα product provided a measure of the reproducibility, quality and yield of each DNA extraction.

For detection of HIV-1 sequences, DNA samples were amplified with 2 different sets of HIV-1 primers: SK145i/SK150 (with SK102 probe) for the *gag* region of the HIV-1; and SK68i/SK69i2 (with SK70 probe) for *env* (92-93). The sequences of these primers were:

SK145i	5'-AGTGGGGGGACATCAAGCAGCCATGCAAA-3'
SK150	5'-TGCTATGTCACTTCCCCTTGGTTCTCTC-3'
SK68i	5'-ttcttIggAGCAGCIGGAAGCACIATGG-3'
SK69i2	5'-ttMatgccCCAGACIGTIAGTTICAACA-3'

I = inosine; M = A + G; low case letters represent extensions to the 5'-end of the modified primers.

Amplification, hybridization and detection of *gag* sequence was performed exactly as described for human HLA-DQα, except the hybridization reaction was in 6 mM NaCl and 4 mM EDTA. Envelope PCR was performed for 30 cycles including denaturation at 95°C for 60 seconds, annealing for 40 seconds at 55°C, and extension at 72°C for 40 seconds, and a hybridization reaction containing 150 mM NaCl.

III.8 DEPLETION OF CD4+ T CELLS AND DIRECT ENRICHMENT OF MONOCYTES

10 ml of citrated whole blood from a HIV-1 seropositive patient was centrifuged at 1000 rpm for 10 minutes. Buffy coat was collected into a 5 ml polypropylene tube (Nunc) and kept on ice. The prepared sample was then subjected to two consecutive incubations with CD4 Dynabeads. Washed CD4 M-450 Dynabeads (250 µl/ml as described above) were added to the isolated buffy coat and incubated for 1/2 hour with gentle rotation at 4°C. After incubation, the tube was put onto the MPC for 2-3 minutes. The supernatant depleted of CD4+ T cells was transferred into a new 5 ml polypropylene tube and depletion of CD4+ T cells was repeated with CD4 Dynabeads (125 µl/ml).

Isolation of monocytes depleted of CD4+ T cells was obtained by direct adhesion to the slide chamber, as described before. Mononuclear cells separated from 10 ml of citrated whole blood without CD4+ T cell depletion were also set up in culture for direct monocyte adhesion for comparison.

IV. RESULTS

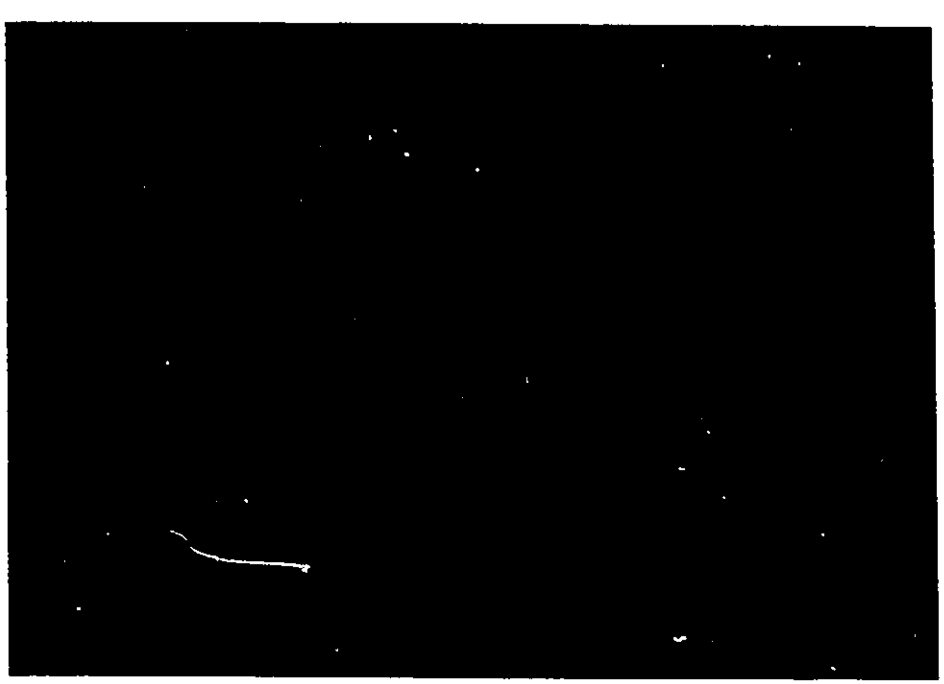
IV.1 DETECTION OF HIV-1 ANTIGEN IN FRESH MONOCYTES FROM HIV-1 SEROPOSITIVE PATIENTS

To investigate if fresh monocytes from HIV-1 infected individuals express HIV-1 antigen, we isolated monocytes by direct adherence to slide chambers, as described in Materials and Methods. Normal donors were also studied as controls. The HIV-1 antigen and cell markers were studied using immunoperoxidase staining. The antibody was a human polyclonal IgG fraction from HIV-1$^+$ donors; it has been tested for its specificity on a HIV-1-uninfected and infected human cutaneous T cell line (HUT-78) (Fig.1). In the HIV-1-uninfected HUT-78 cell line, the cells were negative (Fig.1A), while in the infected HUT-78 cell line, there were cells that stained positive for HIV-1 antigen (Fig.1B). The antibody was also unreactive with a panel of 20 normal donors, which indicates that the antibody did not cross-react with self-antigen.

In HIV-1-infected patients, the monocytes freshly isolated on the slide chamber contained contaminating eosinophils (3%) and it was possible that the positive staining observed was due to eosinophil peroxidase. Eosinophil peroxidase cannot be blocked by incorporating sodium azide into the chromogen (DAB) solutions which were used for inhibition of myeloperoxidase. The use of sodium azide in combination with the more reliable nascent H_2O_2 produced by a glucose oxidase/glucose mixture provides a method for non-deleterious inhibition of the highly resistant form of endogenous peroxidase activity encountered in eosinophil-rich preparations (Fig.2). Thus we could evaluate the presence of HIV-1 antigen without the interference of eosinophil peroxidase staining. We found that fresh monocytes from HIV-1 infected patients did not express measurable amounts of HIV-1 antigen in 10 patients studied.

Figure 1. Specificity of the human polyclonal IgG antibody against HIV-1 antigen. HIV-1 uninfected and infected cells of the human cutaneous T cell line (HUT-78) were stained using an immunoperoxidase method (the anti-HIV-1 antibody was biotinylated in our laboratory and streptavidin-peroxidase was used to label the antibody). Uninfected HUT-78 cells were negative (A), while the infected HUT-78 cells were positive (B) (Magnification 160x).

A



B

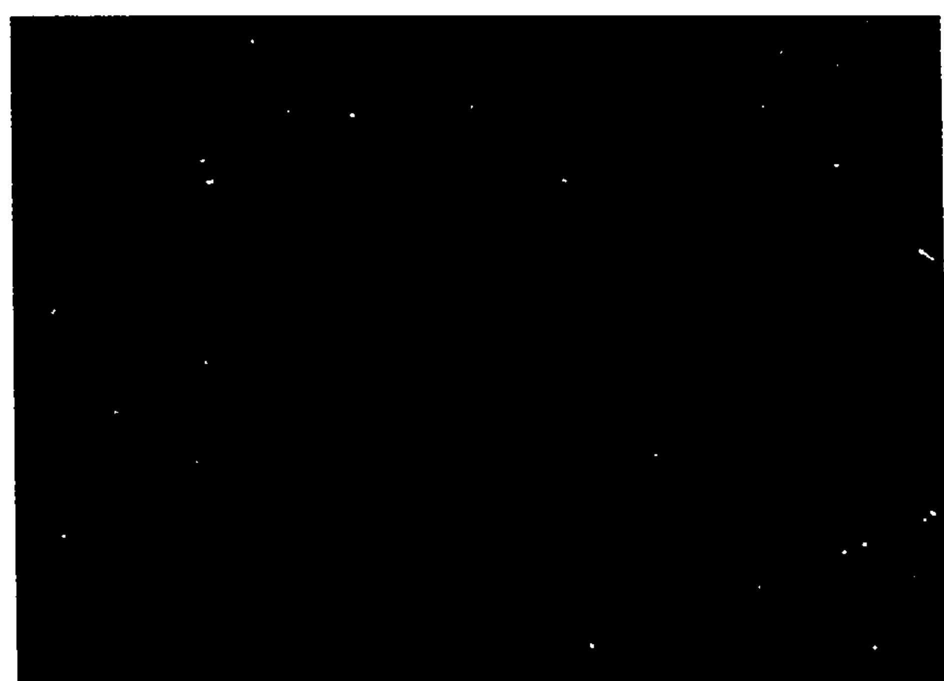
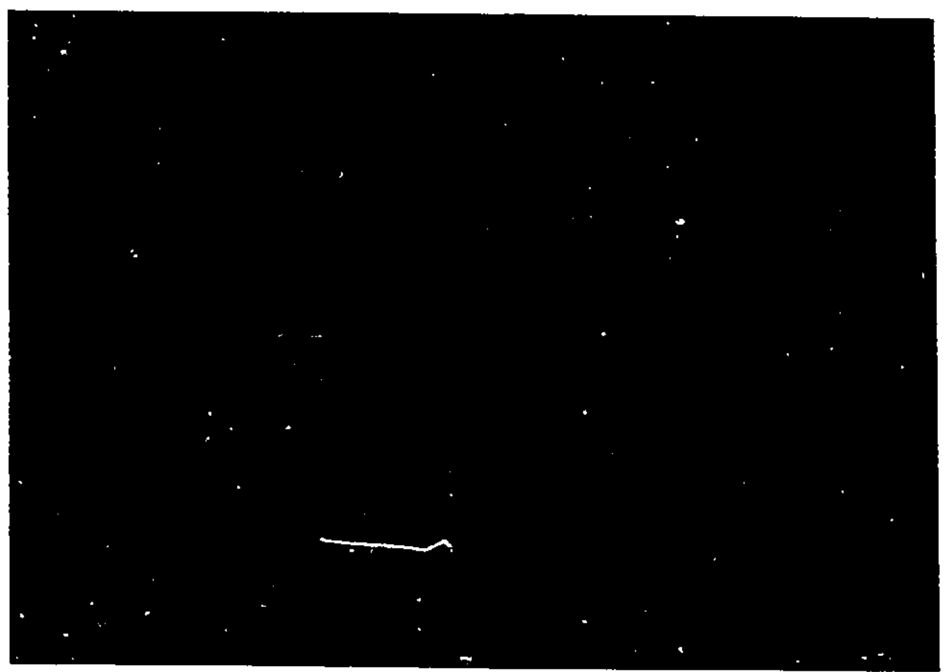


Figure 2. Inhibition of eosinophil peroxidase. Fresh samples from patients with HIV-1 infection contained increased numbers of eosinophils which can be detected with the peroxidase substrate. In the experiment shown in figure 2, eosinophils were detected using only the peroxidase substrate (A), which includes sodium azide to inhibit the endogenous myeloperoxidase of monocytes but not eosinophil peroxidase which is not sensitive to sodium azide. The additon of glucose oxidase/glucose to the substrate/sodium azide mixture generates nascent H_2O_2 that leads to inhibition of both eosinophil and myeloperoxidase (B) (Magnification 160x).

A



B

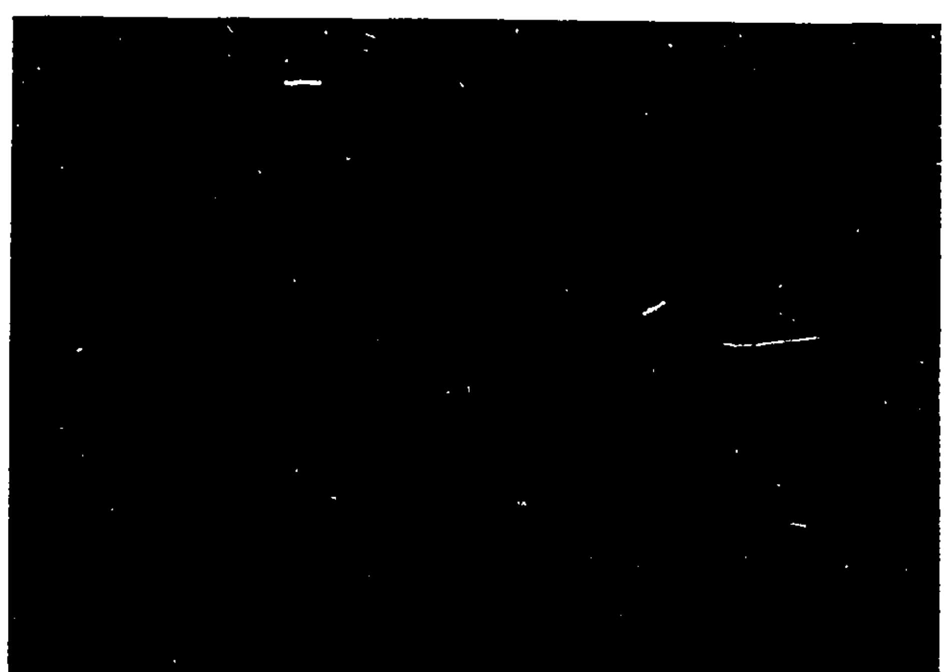
IV.2 DETECTION OF HIV-1 ANTIGEN IN GROWTH FACTORS-STIMULATED ADHERENT MONOCYTES FROM HIV-1 SEROPOSITIVE PATIENTS

To determine the effect of M-CSF, GM-CSF and IL-3 on HIV-1 antigen expression by blood monocytes in culture from asymptomatic HIV-1 positive patients, we also set up monocyte cultures by direct adhesion to a slide chamber vessel in the presence of M-CSF, GM-CSF and IL-3 (Genzyme), as described in Materials and Methods. In the preliminary experiments, we titrated the growth factors, and found that 50U/ml was an optimal concentration for maintaining monocyte survival and maturation in culture. The adherent cells were cultured for 14 days. Again, HIV-1 antigen and cell markers were studied using an immunoperoxidase technique. By counting positive and total cells in various fields of view under the light microscope, we obtained the percentage of positive cells under different experimental conditions.

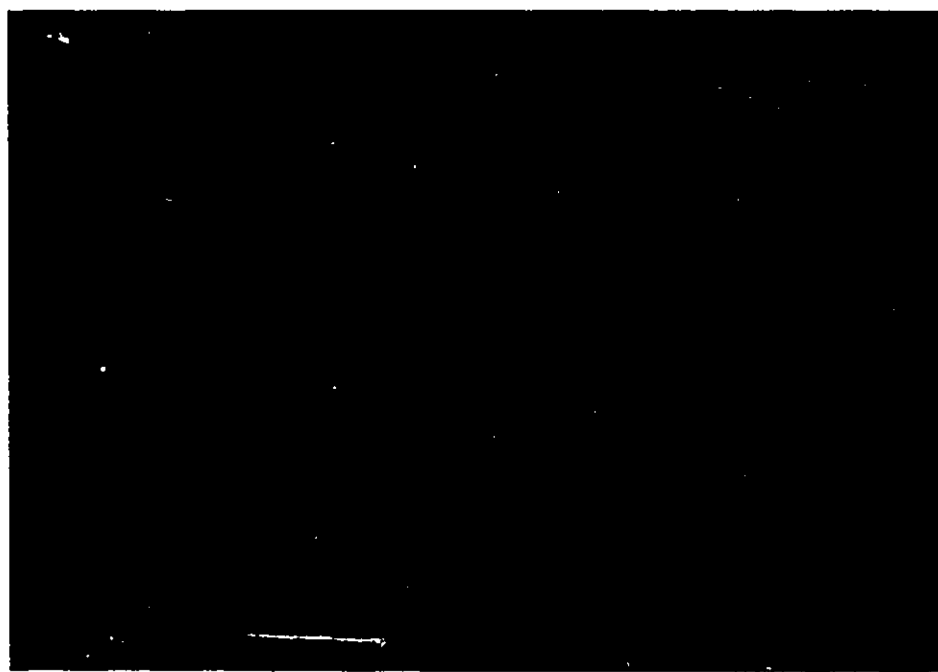
The cultured monocyte surface markers were characterized using anti-CD4 (Leu3a) and anti-CD14 (LeuM3) mAbs (Fig.3). The majority of cells in culture were monocytes (Fig.3A), but there were also a few $CD4^+$ T cells (Fig.3B). It is known that human monocytes are $CD4^+$, but the antigenic density of surface CD4 is much lower than that on $CD4^+$ T cells. As shown in Fig. 3B, the cells which stained as a dark brown ring are $CD4^+$ T cells, while the weak cytoplasmic staining in large cells corresponds to $CD4^+$ macrophages.

We found that after 7 days in culture, monocytes mature to macrophages, some macrophages were HIV-1 positive, but most cells were negative and it was not easy to differentiate positive cells from negative cells, so we decided to do our analysis at 14 days in culture when the HIV-1 antigens were clearly detectable. To analyze the effect of growth factors on the expression of HIV-1 antigen on macrophages from HIV-1 infected patients, we counted the number of positive cells and total number of cells in each well and determined the percentage of HIV-1 positive cells per well for 11 patients studied.

Figure 3. Phenotypic characterization of mononuclear phagocytes in culture. The monocytes from asymptomatic patients were purified using the direct glass adherence procedure as described in Materials and Methods. They were cultured for 14 days and cell-surface markers were characterized by using immunoperoxidase staining with anti-CD4 (Leu 3a) and anti-CD14 (Leu M3) monoclonal antibodies (mAbs). The majority of cells (>90%) stained positive for anti-CD14 (Leu M3) mAb which is a specific cell-surface marker for monocytes (A). There were two cell populations which stained positive for anti-CD4 (Leu 3a) mAb (B). The cells with a dark brown ring were $CD4^+$ T cells (double arrow), while the weak cytoplasmic staining in large cells corresponds to $CD4^+$ macrophages (single arrow) (Magnification 160x).



B



The addition of growth factors showed a trend to slightly higher frequency of HIV-1$^+$ cells in the culture (Fig. 4). However, this trend was only significant for M-CSF ($p<0.002$). But it was very obvious, under the microscope, that the intensity of staining for individual cells was much higher in the culture stimulated with growth factors than in the control culture (Fig.5), indicating increased HIV-1 replication as reported by other investigators (19-21).

IV.3 THE EFFECT OF GROWTH FACTORS ON THE FORMATION OF MGC BY THE ADHERENT MONOCYTES FROM HIV-1 SEROPOSITIVE PATIENTS

The spontaneous formation of MGC was dramatically increased in culture from HIV-1 infected patients when compared to normal control cultures. Since IL-4 has a potent effect in the generation of MGCs, as reported by other investigators (83), we used IL-4 as a positive control to induce MGC formation by normal monocytes. We found that 50U/ml of IL-4 gave the maximum frequency of MGCs from monocytes in culture at day 7 (Fig.6). With the exception of IL-4, the addition of growth factors did not affect the number of MGC formed in cultures from normal donors or HIV-1 seropositive patients (Fig.7).

We also noticed that there was a difference in the number of nuclei per MGC. In order to know if the addition of growth factors would have an effect on the frequency of number of nuclei per MGC, we counted the number of nuclei per MGC from 5 asymptomatic patients and 5 normal donors. These cells were divided into 2 groups, cells that have less than 10 nuclei and cells that have more than 10 nuclei (Fig.8). There was an increase in the number of MGC with >10 nuclei at day 14. Growth factors did not affect the number of nuclei per MGC.

Figure 4. Growth factors induced the expression of HIV-1 antigen by macrophages. The monocytes were separated using the direct glass adherence procedure as described in Materials and Methods and cultured in the presence of growth factors for 14 days. HIV-1^+ macrophages were counted on immunoperoxidase-stained slides. The 4 columns represent the data as mean percentages of HIV-1^+ macrophages per well from 11 HIV-1 seropositive asymptomatic patients. The addition of growth factors showed a trend to slightly higher frequency of HIV-1^+ macrophages in culture. This trend was only significant for M-CSF ($p<0.002$).

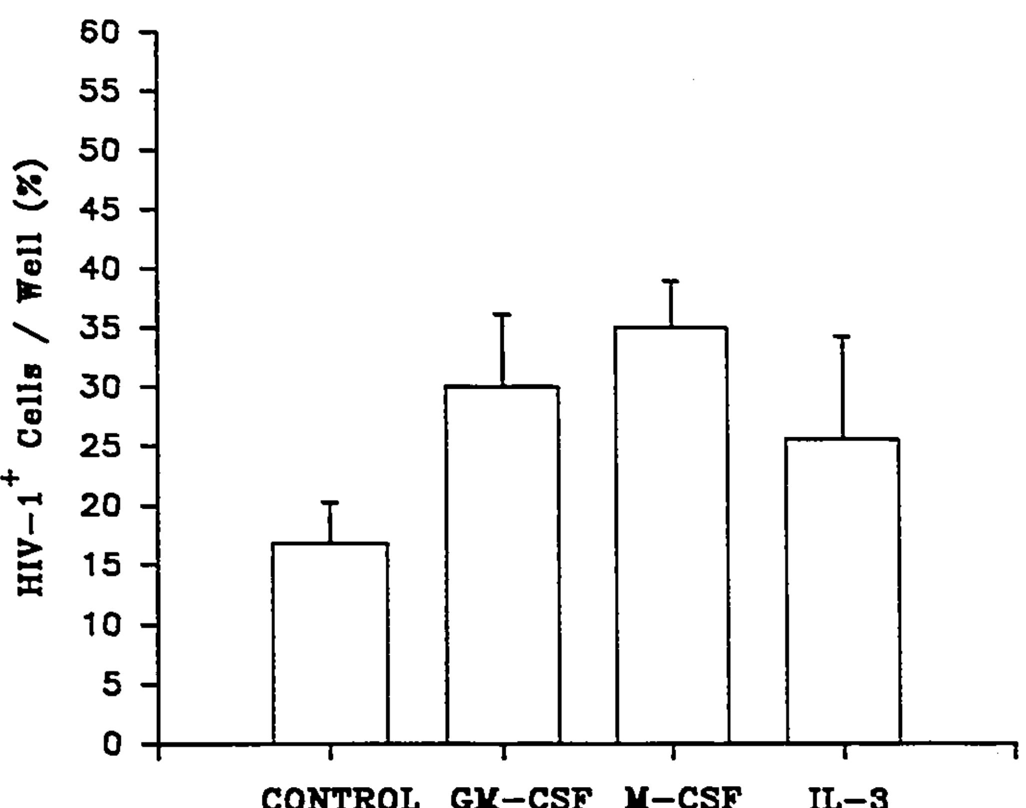
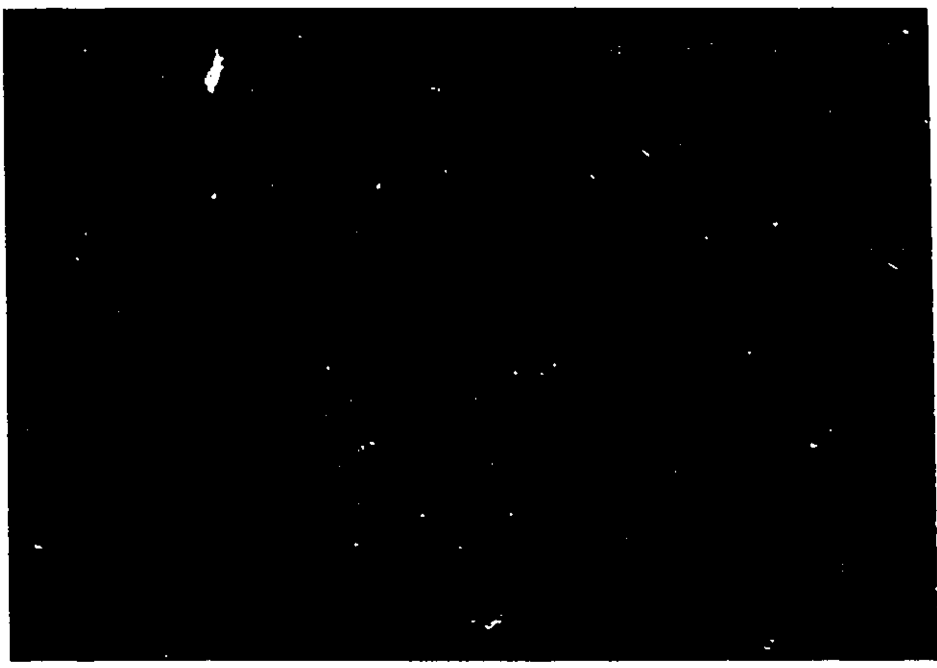

HIV-1+ Cells / Well (%)
60
55
50
45
40
35
30
25
20
15
10
5
0
CONTROL
GM-CSF
M-CSF
IL-3

Figure 5. Effect of growth factors on the expression of HIV-1 antigen by macrophages. The monocytes were obtained using the direct glass adherence method as described in Materials and Methods, and cultured in the presence of growth factors for 14 days. The HIV-1 antigen expression was studied using immunoperoxidase staining with polyclonal IgG antibody from healthy HIV-1^{+} donors. Cells were stained without specific antibody (A), in the absence of growth factors (B), in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (C), and in the presence macrophage colony-stimulating factor (M-CSF) (D). Notice the presence of multinucleated giant cells (MGCs) in the A, B, C, and D (Magnification 320x).

A

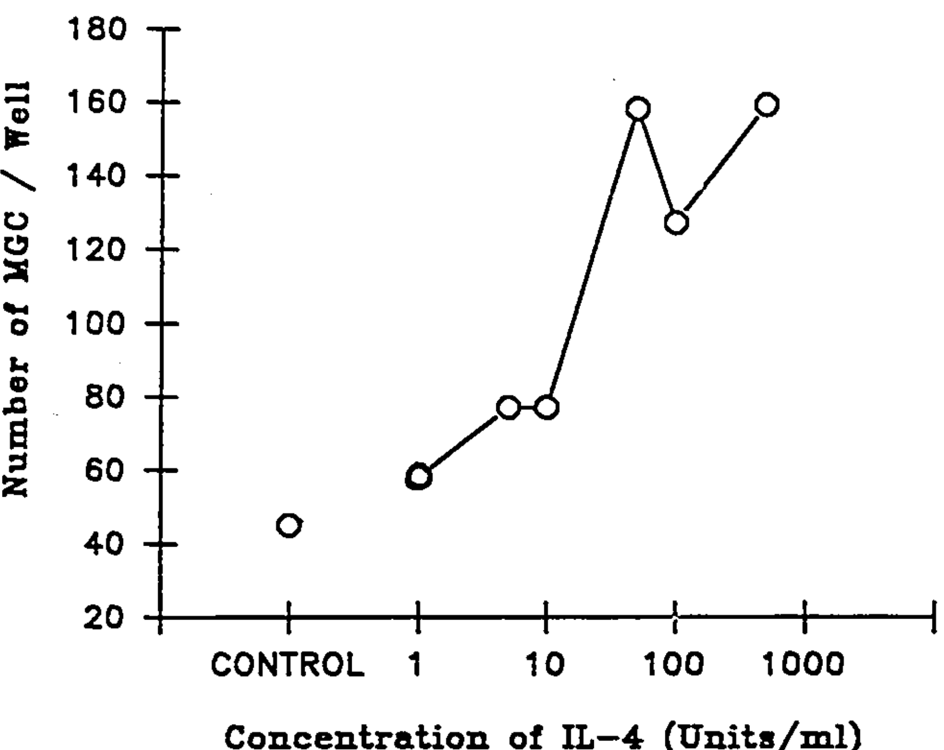


B

C

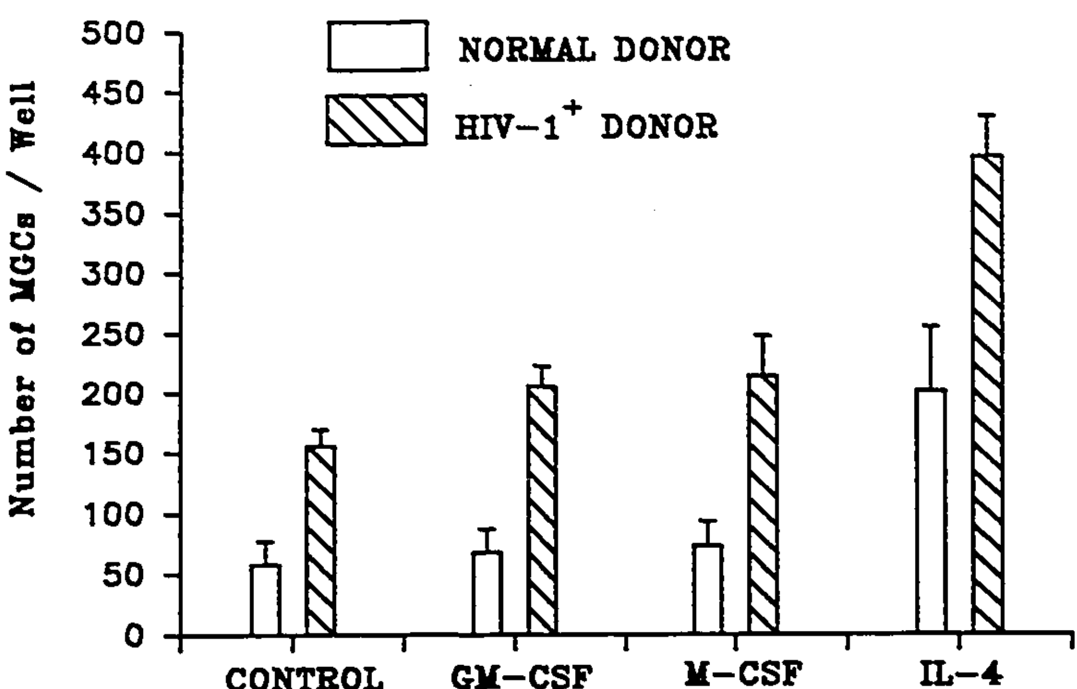
D

Figure 6. Titration of interleukin 4 (IL-4). Dose-response curve for IL-4 induced multinucleated giant cell (MGC) formation at day 7 by normal blood monocytes in culture. 50U/ml of IL-4 gives the maximum frequency of MGCs.



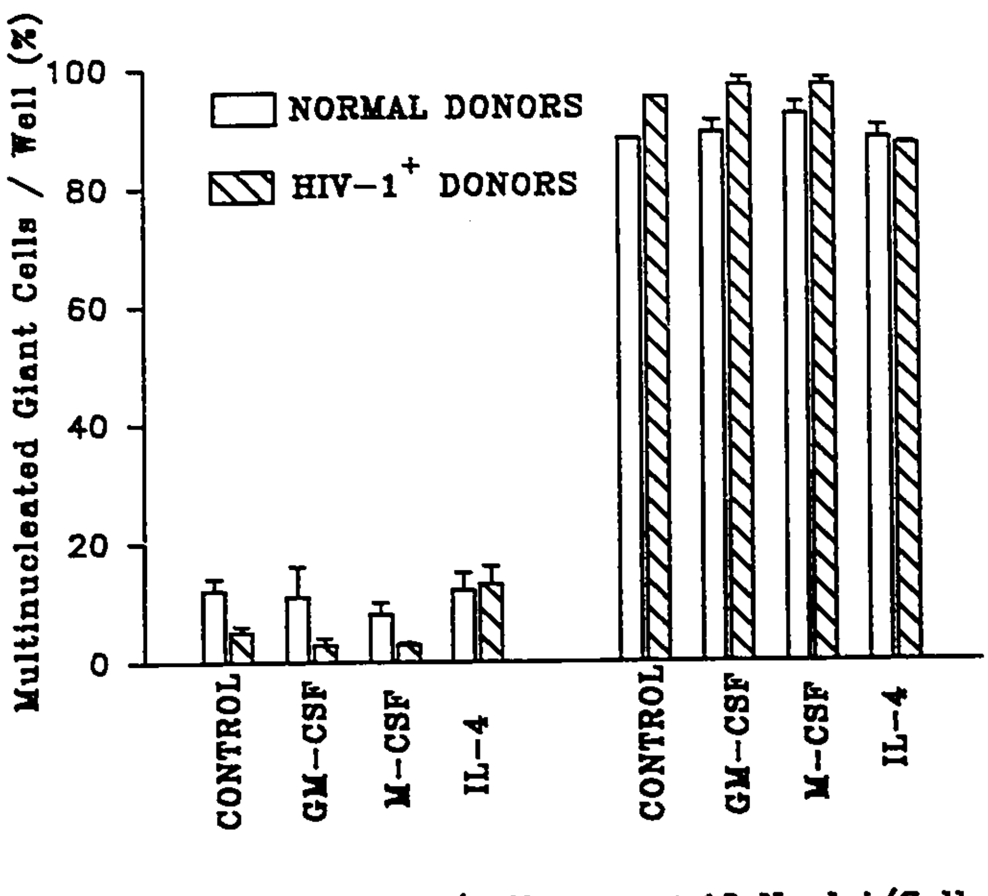
180
160
140
120
100
80
60
40
20
Number of MGC / Well
CONTROL
1
10
100
1000
Concentration of IL-4 (Units/ml)

Figure 7. Effect of growth factors on multinucleated giant cell (MGC) formation. The monocytes were purified by the direct glass adherence procedure and cultured in the presence of growth factors. The MGCs were counted on immunoperoxidase stained slides. The first column of each pair represents mean MGC formation per well in cultures from 5 normal donors, the second column, from 5 HIV-1 seropositive asymptomatic patients. Spontaneous formation of MGCs was increased in cultures from HIV-1 infected patients when compared to HIV-1 seronegative donors ($p<0.05$). With the exception of IL-4 ($p<0.05$), the addition of growth factors did not affect MGC, formed in cultures from both groups ($p>0.05$)



NORMAL DONOR
HIV-1+ DONOR
Number of MGCs / Well
500
450
400
350
300
250
200
150
100
50
0
CONTROL
GM-CSF
M-CSF
IL-4

Figure 8. Effect of growth factors on number of nuclei in multinucleated giant cells (MGCs). The monocytes from 5 positive asymptomatic patients and 5 normal donors were obtained by the direct glass adherence method and cultured in the presence of growth factors. The number of nuclei in MGCs were counted on immunoperoxidase-stained slides. These cells were divided into 2 groups, cells that had less than 10 nuclei, and cells that had more than 10 nuclei. The columns represent the mean percentage of HIV-1^+ MGCs for each group (of total MGCs). There was an increase in the number of MGCs with > 10 nuclei. Growth factors did not affect the number of nuclei per MGC ($p>0.05$)..


Multinucleated Giant Cells / Well (%)
100
80
60
40
20
0
NORMAL DONORS
HIV-1+ DONORS
CONTROL
GM-CSF
M-CSF
IL-4
CONTROL
GM-CSF
M-CSF
IL-4
5-10 Nuclei/Cell
>10 Nuclei/Cell

IV.4 THE EFFECT OF GROWTH FACTORS ON THE EXPRESSION OF HIV-1 ANTIGEN IN MGCs FORMED BY MONOCYTES FROM HIV-1 SEROPOSITIVE PATIENTS

To determine whether the presence of growth factors had an effect on the frequency of HIV-1 positive MGCs, we counted the number of HIV-1 positive MGCs and the total number of MGCs in each well and calculated the mean percentage of HIV-1$^+$ MGC per well for 11 patients, as shown in Fig. 9. Approximately 80% of the MGCs expressed the HIV-1 antigen. There was a trend to higher frequency of HIV-1$^+$ MGCs with GM-CSF and M-CSF; however, this trend was not statistically significant ($p>0.05$).

IV.5 DEPLETION OF CELL SUBSETS USING ANTIBODY-COATED MAGNETIC BEADS

Cell-surface marker analysis of freshly adherent monocytes confirmed that up to 20% of the cells were lymphocytes, mostly T cells (data not shown). Some T cells may persist in culture as shown in Fig. 3B with immunoperoxidase staining. In order to isolate highly purified monocyte fractions and to avoid contamination with HIV-1 infected CD4$^+$ T cells, we did CD4$^+$ T cell depletion experiments. The depletion of CD4$^+$ T cells was done by using mAb coated magnetic beads (CD4-Dynabeads). The flow cytometric analysis of undepleted and depleted samples showed that selective depletion of CD4$^+$ T cells occurred with CD4 Dynabeads (Fig.10). To obtain a more complete depletion of CD4$^+$ T cells, two consecutive incubations with CD4 Dynabeads were employed. Table 5 shows the results of depletion of experiments for 8 patients and 1 normal donor. The depletion of CD4$^+$ T cells from either whole blood mononuclear cells or buffy coat was efficient, ranging from 95-99% in 7 cases out of 9. To further establish the

Figure 9. Effect of growth factors on the frequency of HIV-1$^+$ multinucleated giant cell (MGC) formation. The monocytes were separated by the direct glass adherence procedure and cultured in the presence of growth factors for 14 days. The HIV-1$^+$ macrophages were counted on immunoperoxidase-stained slides. The 4 columns represent the mean percentage of HIV-1$^+$ MGCs (of total MGCs) in each well for 11 patients. This trend was not statistically significant ($p>0.05$).

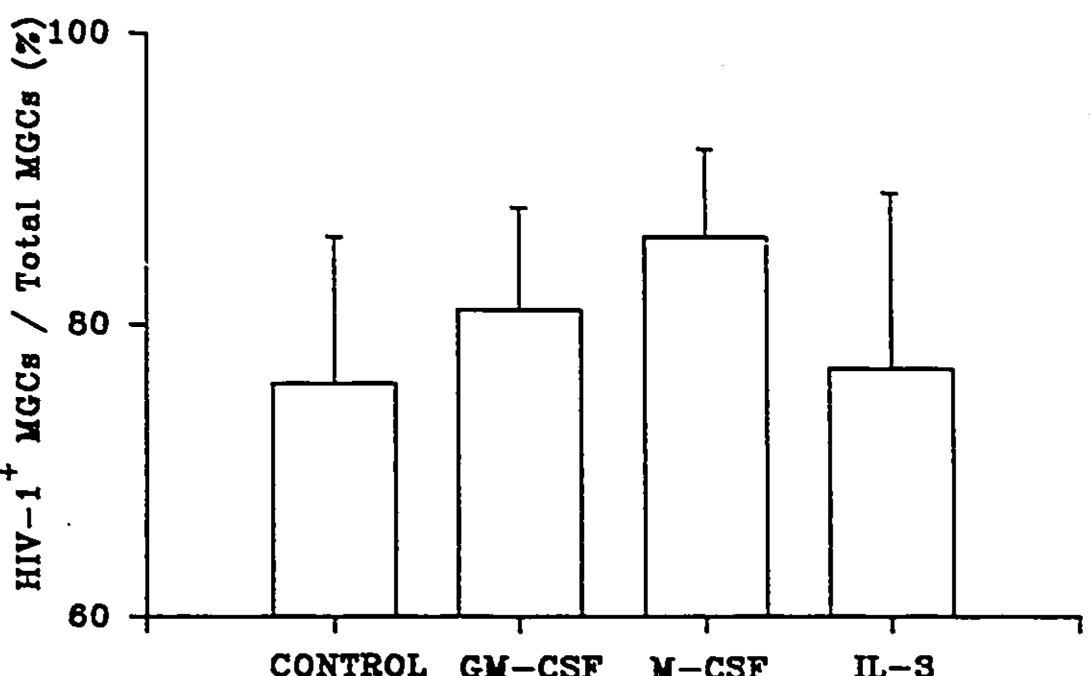
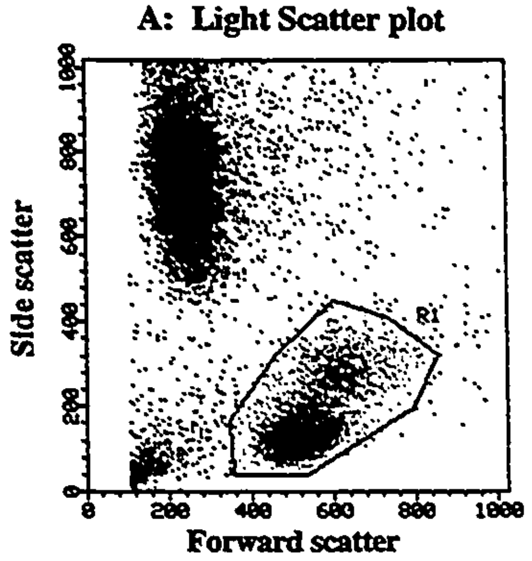

HIV-1+ MGCs / Total MGCs (%)
100
80
60
CONTROL
GM-CSF
M-CSF
IL-3

Figure 10. Depletion of CD4$^+$ T cells from whole blood with magnetic beads.

This figure illustrates the use of anti-CD4 monoclonal antibody(mAb)-coated-Dynabeads to deplete CD4$^+$ T cells from whole blood from a normal donor. The depletion of CD4$^+$ T cells was evaluated by flow cytometry with phycoerythrin-labelled (PE) mAb against CD4$^+$ T cells. The gate was drawn around the light-scatter of lymphocyte and monocyte populations and excluded granulocytes and debris (A). The gated cells were analysed and results presented as a single histogram where the X-axis represents the fluorescence intensity of PE-labelled anti-CD4 antibody and Y-axis is the number of cells that were analysed. CD4$^+$ T cells and and CD4$^+$ monocytes can be distinguished by their fluorescence intensity. There are 3 cell populations in the single histogram (B). The first cell population is negative for CD4 markers. The second which includes dimly stained cells corresponds to CD4$^+$ monocytes (20%). The third, which contains brightly stained cells is related to the CD4$^+$ T cells (42%). After incubation of whole blood with anti-CD4 mAb-coated Dynabeads, the CD4$^+$ T cells were selectively depleted (<0.5%), while CD4$^+$ monocytes still remained (30%) (C).


A: Light Scatter plot
Side scatter
Forward scatter
R1

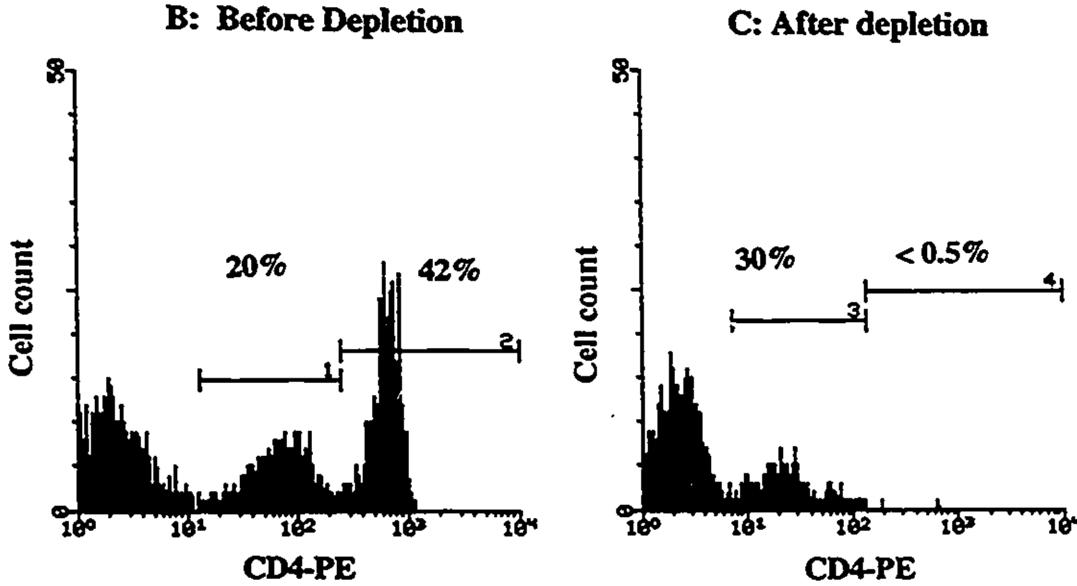

B: Before Depletion
C: After depletion
Cell count
20%
42%
30%
< 0.5%
CD4-PE
CD4-PE

TABLE 5

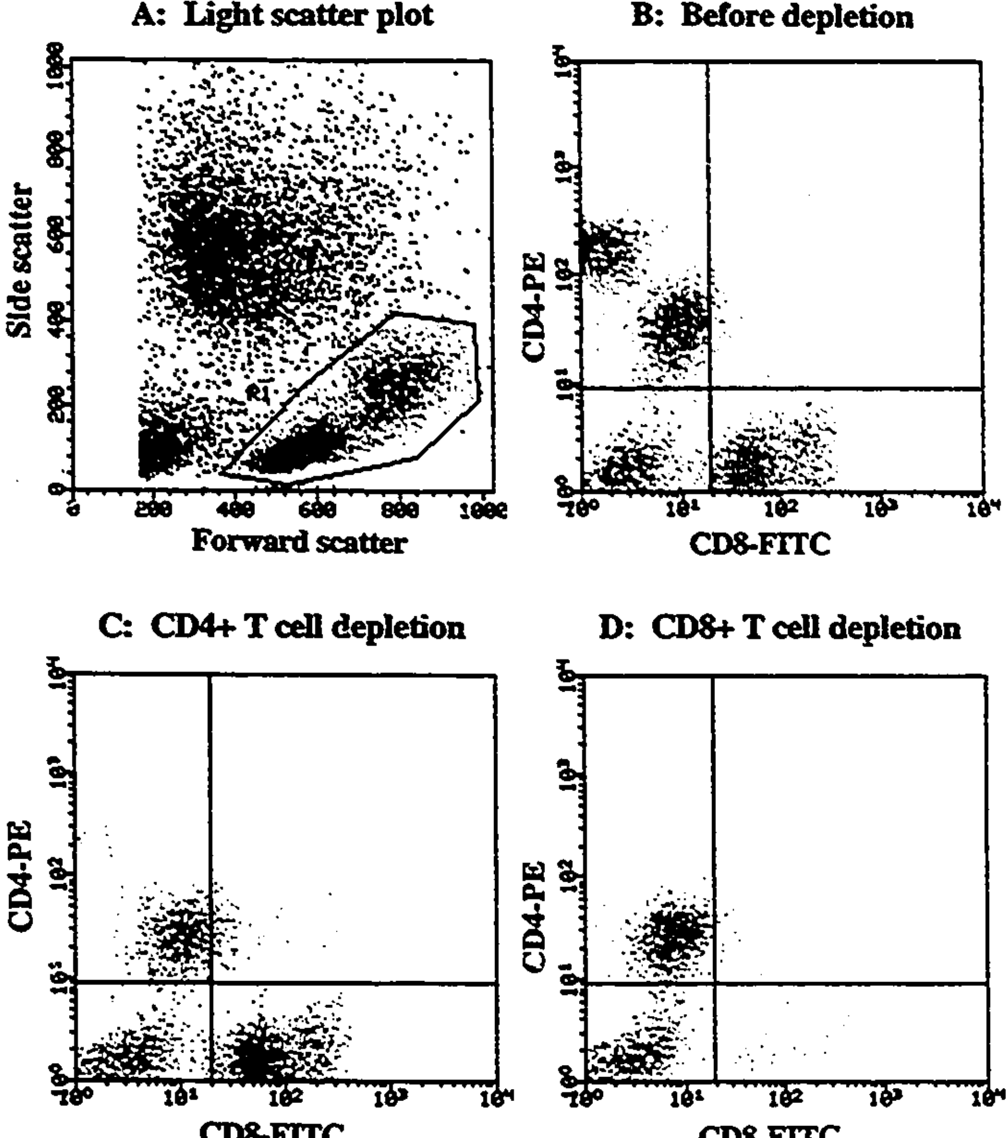
$CD4^+$ T cell depletion from whole blood using magnetic beads

Donors	Control	CD4+ T cells (%)* 1st. Depl.	CD4+ T cells (%)* 2nd. Depl.	% Depletion
MNCs (Ficoll)				
1.	24	nd***	2	92
2.	14	nd	0.1	99
3.	13	nd	0.2	99
4.	13	nd	0.3	98
5.	24	nd	0.2	99
6.**	39	1.5	0.5	99
Blood Buffy Coat				
7.	19	1.4	0.7	96
8.	19	2	1	95
9.	23	16	4.5	80

* = percentage of positive cells in the light-scatter gate drawn around lymphocytes
** = normal donor
***nd = not done

specific cell types infected with HIV-1 in vivo, we purified the whole blood into 3 enriched populations: $CD4^+$ T cells, $CD8^+$ T cells, and monocytes. This was facilitated by sequential depletion using the Dynalbeads coated with different mAbs. First, whole blood from a HIV-1 seropositive patient was sequentially depleted of $CD4^+$ T cells, $CD8^+$ T cells, $CD2^+$ T cells and $CD19^+$ B lymphocytes. CD2 also depleted most NK cells and CD19 depleted B cells. Then the lymphocyte depleted whole blood was layered over Ficoll-Hypaque. The mononuclear cells were collected, and they were highly-enriched monocytes. The purity of each fraction was also evaluated by FACScan flow cytometry with fluorescent-labelled mAbs against T cell subsets. Typical two-color immunofluorescence dot plots are shown in Fig. 11. This figure shows the effect of sequential depletion of $CD4^+$ T cells and $CD8^+$ T cells with Dynabeads in an asymptomatic HIV-1^+ patient. The gate was set up on both lymphocyte and monocyte populations in the light-scatter (Fig.11A). The $CD4^+$ T cell and monocyte populations can be separated by their fluorescence intensity as shown in Fig.11B. In quadrant 1 of Fig 11B, we can see 2 populations of $CD4^+$ cells. In previous experiments we have determined that the lower population consists of $CD4^+$ monocytes and the higher population consists of $CD4^+$ T cells. After depletion with anti-CD4 Dynabeads, we can observe in Fig.11C that the higher T cell population has disappeared while the monocyte population remains. The anti-CD4 mAb coated on Dynabeads has low affinity or does not react with monocytes. It has been reported by others that some anti-CD4 mAbs react poorly with monocytes (43). The next step was depletion of $CD8^+$ T cells, As shown in Fig.11D, the population of $CD8^+$ T cells seen in quadrant 4 of Fig.11B and 11C has disappeared (0.32%). The data showed that depletion with antibody-coated

Figure 11. Whole blood depletion of CD4$^+$ and CD8$^+$ T cell subsets. This figure is an example of sequential depletion from whole blood of CD4$^+$ and CD8$^+$ T cell subsets, from a HIV-1 seropositive patient, using antibody coated Dynabeads. The two-color immunofluorescence dot plots showed the effect of sequential depletion of CD4$^+$ T cells and CD8$^+$ T cells with Dynabeads. The light-scatter gate was set up to encompass both lymphocytes and monocytes and to exclude granulocytes and debris (A). The gated cells were then analysed and results presented as a dot plot graph where the X-axis is the fluorescence intensity of fluorescein (CD8$^+$ T cells) and Y-axis is the fluorescence intensity of phycoerythrin (PE, CD4$^+$ T cells). There are 2 populations of CD4$^+$ cells in quadrant 1 of (B). The lower population consists of CD4$^+$ monocytes and the higher population consists of CD4$^+$ T cells. After depletion with anti-CD4 Dynabeads, the higher T cell population has disappeared while the monocyte population remains(C). In the subsequent step, CD8$^+$ T cells were depleted by anti-CD8 Dynabeads (D), and the population of CD8$^+$ T cells seen in quadrant 4 of (B) and (C) can no longer be seen as shown in quadrant 4 of (D).


A: Light scatter plot
B: Before depletion
Side scatter
Forward scatter
R1
CD4-PE
CD8-FITC
C: CD4+ T cell depletion
D: CD8+ T cell depletion
CD4-PE
CD8-FITC
CD4-PE
CD8-FITC

Dynabeads is an effective method to isolate purified populations of blood cell subsets. In preliminary experiments, we observed poor monocyte recovery for blood samples anticoagulated with heparin. To investigate if the anticoagulant has an effect on the monocyte recovery after depletion of lymphocytes from whole blood, we collected the blood samples from normal donors in both citrate (ACD)-and heparin-containing tubes. As analyzed by FACScan flow cytometry with fluorescence-labelled monoclonal antibodies against T cell subset surface markers in 2 experiments (Table 6), the recovery of monocytes after $CD4^+$ T cell depletion in heparinized blood was much lower (55, 87%) than in citrate collected blood samples (100, 100%). We concluded that citrate-containing blood samples give better recovery of monocytes after depletion of $CD4^+$ T cells.

Table 7 shows the phenotypic analysis of 6 representative examples of sequential isolation of T cell subsets. The monocyte percentage was derived from $CD4^+$ monocytes. The table shows the relative percentage of cells before and after depletion of $CD4^+$ and $CD8^+$ T cell subsets from whole blood. The increased percentage of $CD8^+$ T cells after depletion of $CD4^+$ T cells and that of monocytes after depletion of $CD4^+$, $CD8^+$ T cell subsets was due to the decreased number of total cells; therefore, the relative percentage had increased. Isolated cells were then used in the experiment described in Section IV. 7.

IV.6 THE EFFECT OF $CD4^+$ T CELL DEPLETION ON HIV-1 ANTIGEN EXPRESSION AND MGC FORMATION BY BLOOD MONOCYTES IN CULTURE

To determine the effect of HIV-1 infected $CD4^+$ cells on HIV-1 antigen expression in blood monocytes in culture, we set up parallel monocyte cultures: monocyte cultures depleted of $CD4^+$ T cells and non-depleted monocyte cultures as control. Both monocytes depleted of $CD4^+$ T cells and non-depleted monocytes were obtained by direct adhesion in slide chambers, as

TABLE 6

Depletion of $CD4^{+}$ T cells in both citrated (ACD) and heparinized blood samples

	$CD4^{+}$ T cells			$CD4^{+}$ Monocytes		
	before	after	% depletion	before	after	% recovery
Hep* exp.1	19	0	98	31	17	55
exp.2	63	17	73	87	76	87
ACD**exp.1	31	1	98	26	26	100
exp.2	54	7	87	86	90	100

* = heparinized blood samples
** = citrated blood samples

TABLE 7

Phenotypic analysis of blood cell subsets

		Whole Blood Cell Phenotype (%)*		
		Before	After Sequential Depletion of	
Patient**	Antigen		CD4 T Cells	CD8/CD2,19 Cells
6.	$CD4^+8^-$	9	0.47	0.14
	$CD4^-8^+$	17	16	0.32
	Mo***	14	12	18
7.	$CD4^+8^-$	21	1	1
	$CD4^-8^+$	47	65	4
	Mo	11	5	8
10.	$CD4^+8^-$	25	1	0.41
	$CD4^-8^+$	19	34	3
	Mo	28	26	41
12.	$CD4^+8^-$	18	2	2
	$CD4^-8^+$	34	41	7
	Mo	12	13	28
13.	$CD4^+8^-$	25	1	0.12
	$CD4^-8^+$	34	42	6
	Mo	19	19	23
14.	$CD4^+8^-$	27	14	4
	$CD4^-8^+$	33	43	1
	Mo	24	25	55

* = percentage of positive cells in the light-scatter gate drawn around monocytes and lymphocytes

** = patients number correspond to those in Fig. 15

*** = the monocyte percentage was derived from $CD4^+$ monocytes

described in Materials and Methods. The adherent monocytes were cultured for 14 days. The expression of HIV-1 antigen by macrophages in both $CD4^+$ T cell depleted and non-depleted cultures was studied by immunoperoxidase staining. In cultures depleted of $CD4^+$ T cells (3 patients), with depletion greater than 98%, macrophages did not show detectable HIV-1 antigen expression by immunoperoxidase staining, while in non-depleted monocyte cultures, macrophages did express HIV-1 antigen as in our previous experiment (Fig. 12A and B, Table 8).

Our initial studies revealed that the number of MGC was greatly increased in cultured monocytes from HIV-1 infected patients. To determine whether the MGC formation was due to primary infection of monocytes in vivo or to secondary infection of macrophages by HIV-1-infected $CD4^+$ T cells in vitro, we also evaluated MGC formation in both $CD4^+$ T cell depleted and non-depleted monocyte cultures for 3 patients. Fig. 12C and D represent MGC formation after 14 days in culture in both $CD4^+$ T cell depleted and non-depleted monocyte cultures from one asymptomatic HIV-1^+ patient. The MGCs were visualized by Wright-Giemsa staining. It appeared that there was a dramatic decrease of MGC formation in cultures depleted of $CD4^+$ T cells (Fig. 12D) when compared to the non-depleted monocyte cultures (Fig. 12C). We counted the total number of MGCs in each well from 3 patients as shown in Table 8, and the number of MGCs was significantly decreased after depletion of $CD4^+$ T cells.

IV.7 DETECTION OF HIV-1 PROVIRAL DNA IN BLOOD CELL SUBSETS FROM HIV-1 INFECTED PATIENTS USING A PCR TECHNIQUE

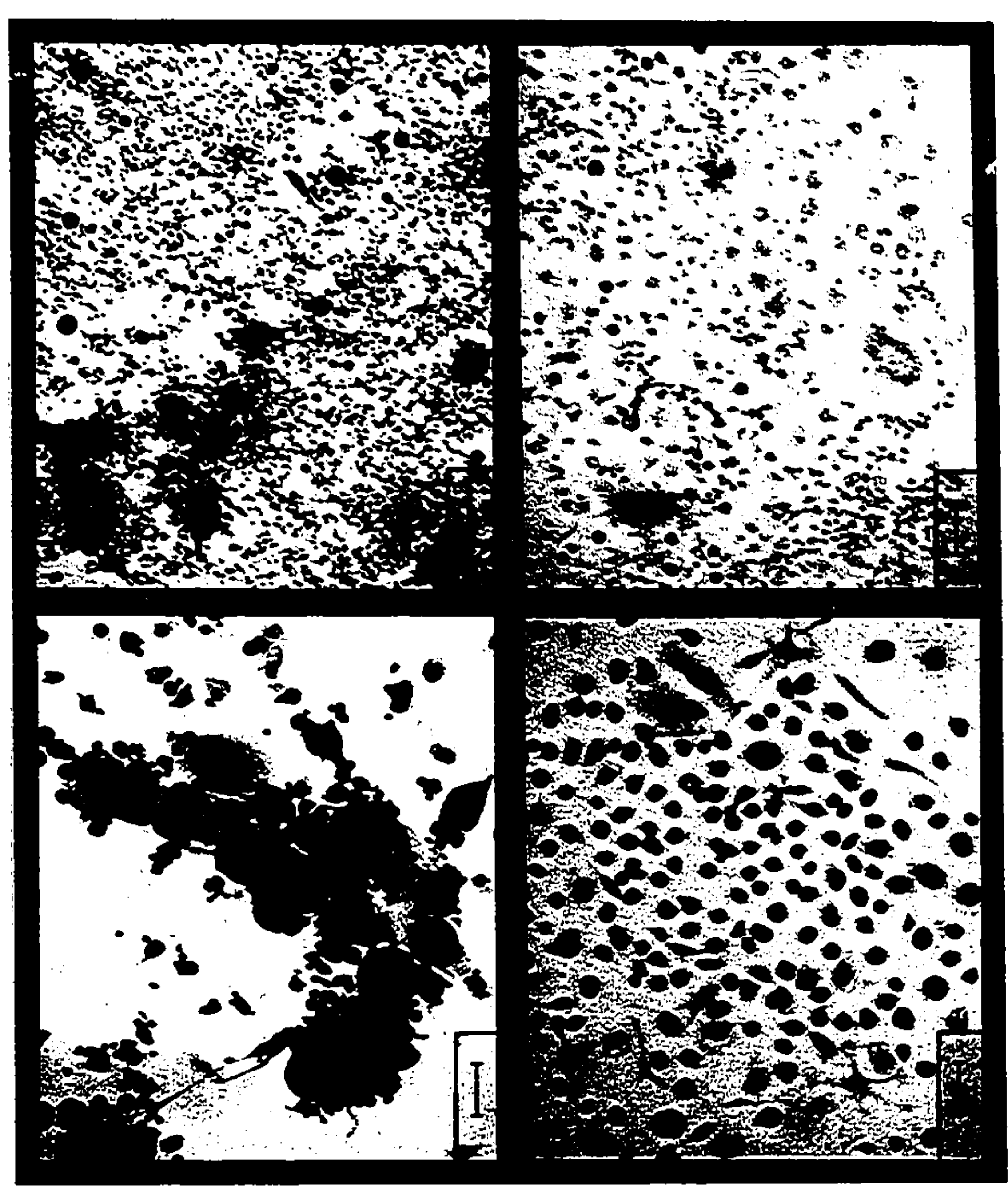
To determine whether monocytes in the peripheral blood of patients contain HIV-1 proviral DNA, and to confirm that the $CD4^+$ cells are a major reservoir for HIV-1 in the peripheral blood, we separated whole blood from 14 patients into $CD4^+$ T cell, $CD8^+$ T cell and monocyte fractions using magnetic beads. These fractions were then analyzed by PCR for the

Figure 12. HIV-1 antigen expression and multinucleated giant cell formation by macrophages before and after CD4$^+$ T cell depletion. Monocytes from CD4$^+$ T cell depleted and non-depleted samples, from an HIV-1 seropositive asymptomatic patient, were separated by the direct adherence method on slide chambers as described in Material and Methods and cultured for 14 days with growth factors. The HIV-1 antigen expression on macrophages was studied using immunoperoxidase staining with polyclonal anti-HIV-1 antibody and MGC formation was evaluated on Wright-Giemsa stained slides or in the immunoperoxidase stained slides.

HIV-1 antigen: In cultures depleted of CD4$^+$ T cells, with depletion greater than 98%, macrophages did not express HIV-1 antigen at detectable levels (B), while in non-depleted monocyte culture, macrophages did express HIV-1 antigen as in our previous experiment (A). In (A), the majority of cells (approximate 90%) are macrophages. However, the small cells without visible cytoplasm at this magnification are lymphocytes. In (B), there are very few lymphocytes. (The unit of measurement in lower left is 10μm. Magnification 25x).

MGC formation: There was a dramatic increase of MGC formation in cultures without depletion of CD4$^+$ T cells (C) when compared to the monocyte culture depleted of CD4$^+$ T cells (D) (The unit of measurement in lower left is 10 μm. Magnification 50x).

A B



C D

TABLE 8

HIV-1 antigen expression and multinucleated giant cell (MGC) formation by macrophages before and after depletion of $CD4^+$ T cells

	HIV antigens		No. MGC* / well	
Patient No.*	Control	CD4 T cell depleted**	Control	CD4 T cell depleted*
2	+	-	30	0
3	+	-	44	0
6	+	-	620	2

* = patient number correspond to those in figure 14

** = depletion of $CD4^+$ T cells was greater than 98%

presence of HIV-1 proviral DNA. For each HIV-1 infected patient that was examined by PCR, 2 different sets of HIV-1 primers were used: SK68i/SK69i2 (with SK70 probe) for *env*, and SK145i/SK150 (with SK102 probe) for *gag*. Each sample was initially analyzed with HLA-DQα to determine whether the sample contained equivalent amounts of DNA in each cell fraction for amplification (data not shown).

We used the 8E5/LAV human $CD4^+$ T cell line as a positive control. Each cell contains a single integrated copy of HIV-1 proviral DNA directing synthesis of defective virus particles (94). We made a serial dilution of 8E5 cell lysates with normal donor MNC lysates to adjust to 25, 12.5, 6 and 3 proviral DNA copies per 12.5 μl, and the diluted samples were subjected to PCR analysis, as shown in Fig. 13. The lowest number of HIV-1 DNA copies detected in our assay was 3 copies. A normal donor MNCs were also run as a negative control in each experiment.

HIV-1 proviral DNA was detected using two sets of primers and probes in $CD4^+$ T cell enriched fraction in all patients examined (Fig. 14). In one case, *gag* was strongly positive, but *env* was negative. In 11 of 14 patients (79%), the monocyte fraction was HIV-1 proviral DNA negative, while in the remaining 3 patients, the monocytes were positive for HIV-1 proviral DNA. The $CD8^+$ T cell fraction was positive for one of the primers, but not for the others in 6 cases.

Figure 13. Standards for HIV-1 proviral DNA detection by Polymerase Chain Reaction (PCR). We have used DNA extracted from the cell line 8E5/LAV. This cell line is a human CD4+ T cell line that has been selected because it contains 1 copy of proviral HIV-1 DNA per cell directing the synthesis of defective viral particles. The DNA concentration has been adjusted so that each sample contained 25, 12.5, 6 and 3 copies of HIV-1 DNA per 12.5μl of sample. DNA extracted from normal blood leukocytes was used as carrier DNA. The samples were then subjected to PCR analysis with sets of primers for *gag* and *env* as described in Materials and Methods (the exposure time for *env* is 21 hours, for *gag* 1 hour). The minimum number of copies detected was 3 copies. .

HIV-1 STANDARDS

Primers	No. of copies				
	25	12.5	6	3	Neg.
ENV					
GAG					

Figure 14. HIV-1 proviral DNA analysis by PCR of blood cell subsets from 14 HIV-1 infected seropositive patients. 4 ml of whole blood from 14 HIV-1 seropositive patients were separated into $CD4^+$ T cells, $CD8^+$ T cells and monocyte fractions by magnetic beads as described in Materials and Methods. The PCR analysis was done on 25 µl of cell lysate, equivalent to 1.5×10^5 cells for each primer set. Amplification of HIV-1 DNA was demonstrated by using 2 different sets of HIV-1 primers: SK145i/SK150 (with SK 102 probe) for *gag* region; and SK 68i/69i2 (with SK 70 probe) for *env*. The amplified products were hybridized with ^{32}P-labelled probes and analyzed on a 10% polyacrylamide gel. Positive hybridization product was identified by exposure to film. HIV-1 proviral DNA was detected in $CD4^+$ T cell enriched fraction in all patients examined. In 11 of 14 patients (79%), the monocyte fraction was HIV-1 proviral DNA negative, while in the remaining 3 patients, the monocytes were positive for HIV-1 proviral DNA. The $CD8^+$ T cell fraction was positive for one of the primers, but not for the others in 6 cases.

* The $CD4^+$ count was the relative percentage acquired from the whole blood and analyzed by FACScan flow cytometry by gating on both lymphocytes and monocytes in light-scatter.

	GAG			ENV			CD4 counts*
	CD4	CD8	Mo	CD4	CD8	Mo	(%)
1							11
2							2
3							17
4							24
5							nd
6							9
7							21
8							6
9							nd
10							25
11							19
12							18
13							25
14							27

V. DISCUSSION

V.1 MONOCYTE CULTURES AND HIV-1

In the first part of our studies, our objectives were (i) to study the effect of HIV-1 infection and growth factors on the expression of HIV-1 antigen by blood mononuclear phagocytes in culture from HIV-1 infected patients, (ii) to study the effect of HIV-1 infection and growth factors on the formation of monocyte-derived multinucleated giant cells (MGC). We showed that when monocytes were purified using the standard glass adherence procedures, the monocytes mature to macrophages in vitro and after 14 days in culture, they become positive for HIV-1 antigen expression (11 of 11 patients). The growth factors GM-CSF, M-CSF, and IL-3 increased the level of HIV-1 antigen expression per cell (Fig.5) and only M-CSF appeared to increase the number of HIV-1$^+$ macrophages (Fig.4). We also observed that in HIV-1 infected mononuclear phagocyte cultures, there were spontaneous formation of MGC without addition of IL-4 (Fig.7). The addition of growth factors did not affect the MGC formation or the number of nuclei per MGC (Fig.8). Our initial results appeared to confirm what was already reported by others (19-21). However, the mechanism for hemopoietic growth factors enhancing HIV-1 production in mononuclear phagocytes is currently unknown. The mechanism may relate directly to the increase in proliferative capacity of this lineage. In vivo, GM-CSF, M-CSF and IL-3 are important regulators of hematopoiesis. GM-CSF and IL-3 are produced by activated T cells and M-CSF is produced by activated macrophages. A large number of monokines and lymphocytokines are involved in regulating the interaction of T cells with mononuclear phagocytes during an immune response. Because HIV-1 can infect T cells and mononuclear phagocytes, interactions between these cells by means of cytokines are likely to be altered and in some instance, HIV-1 replication is affected.

Recent studies challenge the relevance of HIV-1 monocyte tropism as an in vivo phenomenon. Mcelrath et äl (58) have examined HIV-1 production in cultured mononuclear cells

in a large group of patients in various stages of infection. They found that blood mononuclear cells from seropositive individuals are more likely to produce virus as disease progresses and that the primary source of virus is from a $CD4^+$ T cell-enriched fraction. Their data do not implicate the monocyte as a primary or exclusive source of productive HIV-1 infection in any stage of infection because they were only able to demonstrate blood monocyte infection in less than one-third of the patients tested. Our results are in accordance with their finding.

V.2. HIV-1 PROVIRAL DNA IN MONOCYTE-DERIVED MACROPHAGES FROM HIV-1 INFECTED PATIENTS

PCR analysis(22-24) also revealed that in AIDS patients, nearly all HIV-1 DNA was within the CD4-expressing T cells. Limiting dilution analysis was used to show that the frequency of $CD4^+$ T cells expressing HIV-1 DNA was 1:100 (22). However, monocyte infection with HIV-1 is a relatively infrequent event. It was concluded that the most common in vivo circulating reservoir of HIV-1 is the $CD4^+$ T lymphocyte, even in those cases where the isolated viruses showed preferential in vitro tropism for cells of the macrophage lineage.

V.3 A NEW WORKING HYPOTHESIS

The studies discussed above suggested that our initial results demonstrate an in vitro phenomenon rather than the consequence of in vivo infection of monocytes. A flow cytometric analysis of adherent cultured monocytes as well as immunoperoxidase staining of 7 day cultures revealed that in cultures of glass or plastic adherent monocytes there was a substantial contamination with lymphocytes, and that lymphocytes appeared to survive well for at least 7 days. Immunoperoxidase staining showed that a substantial number of the lymphocytes were $CD4^+$ T cells. This observation led to the hypothesis that there were HIV-1 infected $CD4^+$ T cells in the cultures and that the infected cells survived and produced virions in cultures. The

released HIV-1 particles may then have been able to infect surrounding maturing monocytes. In addition, cell-cell contact between T cells and monocytes was very likely because the cultures were set up at a high cell density. This would also favore viral transmission to the maturing monocytes. The use of growth factors may have favored increased viral replication, so that easily detectable expression of HIV-1 antigen was reached by day 14 in all cultures. The presence of growth factors may also have enhanced the survival of, and virus production by, T cells during the first week in culture. However, there is scant data in the literature to support this latter hypothesis; perhaps the culture conditions were just better.

V.4 EFFECT OF CD4$^+$ T CELL DEPLETION ON MONOCYTE CULTURES

It became clear that we had to determine if the presence of HIV-1 particles in day 14 macrophages was due to either true in vivo infection or artificial in vitro infection by contaminating infected CD4$^+$ T cells. To answer this question, we set up two types of experiments. First, we repeated tissue culture experiments before and after depletion of CD4$^+$ T lymphocytes. CD4$^+$ T cells were depleted from whole blood using anti-CD4 mAb-coated magnetic beads. Preliminary experiments using normal blood and blood from patients were done to optimize the procedure. Theoretically, the comparison of absolute cell counts before and after depletion of CD4$^+$ T cells is the most accurate way to evaluate the effectiveness of depletion. We could not do this because the volumes of the patients' samples were limiting. We found that the use of magnetic beads is a rapid and highly efficient method for depletion of CD4$^+$ T cells (Table 5).

It seems that the CD4 molecule on the surface of monocytes is not accessible to anti-CD4 coated Dynabeads. We assumed that the CD4 epitope on monocytes recognized by antibody is altered as compared to that on T cells. This observation has also been made using another anti-CD4 mAb, OKT4 (9). It is also possible that the CD4 molecule on monocytes may not be the same as the CD4 on T cells. This difference may be located on the CD4 region that contains the

epitope recognized by the OKT4 antibody or CD4-antibody-coated magnetic beads, and perhaps result from differences in post-translational modification such as glycosylation. To date, there are no reports in the literature comparing the sequence of CD4 on monocytes and T cells.

For 3 of the patients examined, monocyte cultures were set up both before and after depletion of $CD4^+$ T cells. The culture conditions were the same as in our initial experiments. We found that depletion of $CD4^+$ T cells abrogated the capacity of maturing monocytes to form multinucleated giant cells in culture (Fig. 12C and D). In addition, there was no expression of HIV-1 antigen (Fig. 12A and B). Thus, we concluded that in our 3 cases, monocytes were not infected in vivo, but acquired the infection in vitro, most likely from contaminating, infected $CD4^+$ T cells. However, it is possible that some monocytes may have been infected with very few virions, therefore, a longer culture period is needed to show positive staining of HIV-1. It is also possible that although viral entry occurred, there was incomplete viral transcription in similar fashion to what has been reported for resting T cells (54). Latent infection with delayed activation may also account for the negative results. To rule out such explanations, we performed experiments where HIV-1 proviral DNA was detected using a very sensitive method as discussed in the following section. Massari et al (23) have concluded that among PBMC, the most common in vivo source of HIV-1 strains which preferentially infect macrophages in vitro is the $CD4^+$ T lymphocyte. It is also a very clear indication that the presence of infected $CD4^+$ T cells was required to induce MGC formation and most likely is due to HIV-1 infection of differentiated monocytes. It is still unclear whether the increased number of MGCs is due to the syncytia formation mediated by gp120-CD4 interaction, or if HIV-1 infection may trigger the lymphokine-(IL-4)-induced pathway of MGC formation. It has been shown that IL-4 is also a potent enhancer of HIV-1 replication in mononuclear phagocytes (95).

V.5 HIV-1 PROVIRAL DNA IN FRESH MONOCYTES

To confirm our observations and to determine if monocytes have latent infection, we set up a second type of experiment to test for the presence of proviral DNA. We isolated $CD4^+$ T cells, $CD8^+$ T cells and monocytes using mAb-coated magnetic beads. Various preliminary experiments were done to optimize the procedure. We found that leukocyte subsets could be isolated from 4 ml of whole blood with a high degree of purity. The isolated cell subsets were then processed to determine if they contained HIV-1 DNA copies using the PCR amplification technique. HIV-1 DNA primers for both *gag* and *env* genes were used. We used 2 sets of primers to obtain corroboration data and to prevent false negative results due to technical problems such as the presence of inhibitor. Both sets of primers were modifications of previously reported primers (92), and have proven to be very effective in detecting HIV-1 DNA (93). Our findings confirmed that the major cellular reservoir of HIV-1 in blood is $CD4^+$ T cells (Fig. 14). In one case, *gag* was strongly positive, but *env* was negative. This is likely due to viral *env* sequence variation in the patient (92), perhaps occurring in the amplified region. Such mutations may have resulted in an amplified region that could not hybridize with the probe. The $CD8^+$ T cell fraction was positive for one of the primers, but not for the others in 6 cases. By definition, $CD8^+$ T cells are considered PCR negative because the signal is usually weak and it may represent cross-contamination with residual $CD4^+$ T cells or double positive ($CD4^+8^+$) T cells. The most important finding was that the monocyte population was HIV-1 PCR negative in 11 of 14 cases. In the remaining 3 cases, the signal corresponded to 3 to 6 copies when compared to standards run at the same time. Thus, the results may be due to either residual, infected $CD4^+$ T cells that occur with the separation methods used, or actual HIV-1 proviral DNA in monocytes. The studies of Massariet et al (22) and Spear et al (24) have also shown that in some patients monocytes may be infected. In one report (24), repeated testing of blood samples obtained over several months confirmed that monocytes were HIV-1 proviral DNA positive in 1 patient. In our study, the PCR was repeated in a subsequent blood samples in 3 cases with monocytes positive

for HIV-1 proviral DNA, and the second test confirmed the first result. We can thus argue that such patients may have true HIV-1 infection of blood monocytes. According to the CD4 counts, these patients appeared to have an intermediate stage of the disease (18, 25 and 27% CD4 counts, respectively). The significance of HIV-1-positive monocytes in such patients remains to be determined.

Our study indicates that blood monocytes are not infected in most individuals or are infected at relatively low levels. It is possible that sequence variation in monocytotropic strains of HIV-1 could have made amplification and detection less sensitive. This is unlikely because the primers and probes were derived from highly-conserved HIV-1 regions. One of the interesting features in cytomegalovirus (CMV) infection is that impaired monocytes which contain the viral particles are engulfed by polymorphonuclear neutrophils in the circulation (Rossier, personal communication). This may also apply to HIV-1 infection where the HIV-1 infected monocytes may be phagocytosed by neutrophils. However, PCR analysis on neutrophils from HIV-1 infected patients suggested that they do not contain HIV-1 proviral DNA (24). Thus, even if HIV-1 virions are phagocytosed by granulocytes or monocytes, that particular route of entry may lead to destruction of the virus and not to viral replication. In addition, viral entry in monocytes appears to be very low compared to that in T cells, and for that reason, viral replication may take longer to reach detectable levels. Thus, even if monocytes are infected, the number of copies may be very low during their short transit period in the blood.

Recent study indicates that bone-marrow stem cells may be infected in AIDS (86); and that isolated myeloid progenitor stem cells ($CD34^+$) can be infected by HIV-1 in vitro (87). However, two reports (66-67) have demonstrated, using PCR, that $CD34^+$ hematopoietic progenitor cells are not a major reservoir of HIV-1, and committed myeloid and erythroid progenitors from AIDS patients are responsive to hematopoietic growth factors in vitro. Also, expression of HIV-1 proviral DNA only in monocytes but not neutrophils would suggest infection of cells at a stage of development after the common stem cells that gives rise to both cell types (24).

V.6 WHY MONOCYTES ARE NOT USUALLY INFECTED

In early studies, it was shown that virus can be cultured from peripheral blood monocytes (8, 11-12). In addition, macrophages at several tissue sites have been found to express HIV-1 specific antigens and RNA (13, 15, 70). It is possible that virus bound to monocyte-surface CD4 or FcR or CR (59-60) could, in some cases, explain the ability to culture HIV-1 from monocytes. However, many of the reported culture methods require either in vitro maturation of monocytes to macrophages, or treatment with colony-stimulating factors before successful infection can occur, suggesting that monocytes are relatively resistant to infection by HIV-1 (again, probably due to low viral entry in spite of normal viral binding to the CD4 receptor) (8-9, 16, 19-20, 96). Low viral entry may not be related to the quantity of CD4 receptors. Kim et al (62) have shown that in a monocytic cell line selected because they have more CD4 receptors than a T cell line, viral entry was still very low. He postulated that a second step is required for viral entry and that step is not well-developed in monocytes. There is ample evidence for the second step, but the nature of it is unknown. The other possibility is that the CD4 molecule of monocytes may differ from that of T cells, perhaps by post-translational modification. Kim et al also demonstrated that once the virus entered the host cell, reverse transcription, use of the LTR, RNA expression, and production of virus particles were about as efficient in monocytes as in T cells. A low susceptibility to infection of monocytes but not macrophages has also been noted in other lentivirus diseases (97). If monocytes do exhibit a resistance to infection in vivo, then the tissue macrophage may be the actual site where infection occurs.

We want to emphasize that monocytes are not macrophages. Although blood monocytes and tissue macrophages are developed from the same lineage, macrophages represent terminally differentiated cells which have a unique place in the tissue. Macrophages are critically situated in the various tissues, usually close to the microvasculature and surrounding epithelia and mesenchymal cells. When monocytes leave the blood to enter the tissues, they mature to tissue macrophages which are characterized by increasing size, developing pseudopodia, and a

cytoplasm containing abundant granules. The mature macrophage has little peroxides activity but increasing amounts of acid hydrolysis (18). Furthermore, macrophages in different tissues have particular characteristics. They vary in the extent of surface receptors, oxidative metabolism, arachidonate products, and expression of class II MHC molecules.

The half-life of monocytes in the circulation is 1-3 days. This would leave enough time for infection of monocytes while in the peripheral blood. Furthermore, the plasma of HIV-1 infected individuals contains HIV-1 virions, capable of infecting target cells (56). It is possible that some cellular factors, other than the expression of CD4, may be required for efficient HIV-1 infection. These factors may only be induced during the maturation of the monocyte to macrophage. Therefore, the relatively immature monocyte, even in the presence of infectious virus, may be resistant to infection due to the absence of the additional cellular factors.

Our results favor the conclusion that, unlike the $CD4^+$ T cells and tissue macrophages, the relatively immature monocytes found in the peripheral blood are less susceptible to infection by HIV-1. Our results also revealed that when normal monocytes/macrophages were infected in vitro, the viral replication was not detected until after 7-10 days in culture. It seems the susceptibility of the cells to infection is closely linked to the maturation of monocytes to macrophages. Further studies are needed to define the route by which monocytes/macrophages are infected with HIV-1 and the role of macrophages as reservoirs of infection.

V.7 CELLULAR TROPISM

Early reports on HIV-1 isolation have documented target-cell restriction, that is, HIV-1 isolates grew preferentially in either T cells or monocyte-derived macrophages or monocyte cell lines (9, 19, 98). Most laboratory HIV-1 strains have been derived from long-term passage in T cells and T cell lines. Such strains replicate poorly or not at all in macrophage target cells (8-10). In several cases, HIV-1 strains have been isolated from tissue macrophages, particularly, from neural and pulmonary tissues of infected individuals (9, 15-16, 71). These strains appeared to

replicate preferentially in mononuclear phagocytes. In particular, AIDS dementia is highly correlated with infection of macrophages and/or microglia in the brain (13-16). This observation led to the suggestion that there are 2 strains of HIV-1, one grows preferentially in T cells (lymphocytotropic) and the other grows better in macrophages (monocytotropic). Recently Meltzer et al (99) have shown that HIV-1 isolates that had been serially passaged in monocyte-derived macrophages (grown in the presence of M-CSF) grew very well in PH/IL-2 treated normal lymphoblasts. Thus, it appears that there is no target-cell restriction in virus replication for HIV-1 isolates grown in M-CSF treated macrophages. Such isolates grow equally well in lymphoblasts. In subsequent experiments, Meltzer et al (99) studied 5 clinical isolates of HIV-1. In each of the 5 patients, HIV-1 virions were isolated using both M-CSF treated macrophages and PH/IL-2 treated lymphoblasts. The isolates were then passaged in cultures of homologous cell types. After serial passage, they showed that HIV-1 isolates grown in macrophages are capable of infecting lymphoblasts, confirming the early experiments. However, when lymphoblast-isolated HIV-1 was used to infect macrophages, no evidence of virus growth in the M-CSF treated macrophages was observed (by p24 antigen release, reverse transcriptase, or infectious titter). Also, macrophage-passaged HIV-1 isolates that were later on passaged several times in lymphoblasts failed to grow successfully in macrophages. They concluded that there are two species of HIV-1 that are selected by the target-cell used in the isolation procedure. Virions isolated in macrophages are dual tropic; virions isolated in lymphoblasts replicate only in T cells.

They suggested target cell selection of diverse HIV-1 variants may explain the phenomenon of macrophage tropism. Viruses isolated in macrophages retain their ability to infect both macrophages and T cells. When T cells were used to isolate and to passage HIV-1, only those HIV-1 virions capable of infecting T cells were selected and virions capable of infecting macrophages were selected against. The loss of the capacity to infect macrophages by virions selected on T cells may reflect any of several changes in the virus such as mutations in structural or regulatory viral genes; transcriptional or post-transcriptional changes in viral product levels; complementation of gene products of different integrated provers yielding virus with dual

tropism or T cell tropism; and host cell regulatory factors that are permissive only for certain viral variation.

It is also possible that tropism reflects changes induced by the selection during the long-term culture and may not reflect true in vivo tropism. Our findings and those of others (22-24) indicated that the major blood cellular reservoir of HIV-1 are $CD4^+$ T cells and that monocyte-derived macrophages, from HIV-1 infected patients, acquired the virions from T cells and not from infected monocytes. Monocytes themselves are not infected in about 80% of patients.

V.8 A MODEL FOR THE PATHOGENESIS OF HIV-1 INFECTION

HIV-1 is transmitted mainly by sexual contact, by infected blood or blood products, and perinatally by mother to infant. The initial target cells in the rectum, genital tract or blood stream have not been identified. Mononuclear phagocytes are likely candidates since they display the specific function of antigen processing and presentation (Langerhans cells in the skin). It is possible that the $HIV\text{-}1^+$ APC, rich in HLA-DR determinants, migrate to the lymph nodes where they interdigitate with T cells and present antigen to the $CD4^+$ T cells and thus may transmit the virus to them. The virus-infected $CD4^+$ T cells then circulate into the various tissues and transmit virus to the other tissue macrophages and other $CD4^+$ T cells. It has to be further determined why the relatively high frequency of productively infected macrophages found in brain, lymph nodes and lung is not observed in all tissues. The free virus in the plasma may also play a role in infecting the macrophages in highly vascularized tissues (e.g. marrow, spleen).

It appears that, in contrast to HIV-1 infected T lymphocytes from which virions are rapidly released into the medium, the HIV-1 infected macrophages show intercellular storage of viral particles within vesicles. Thus, the macrophages can harbor the virus for a long time without themselves being killed, and may serve as a persistent viral reservoir. In addition, macrophages may differ from T cells in their intercellular metabolism of drugs and their reaction to lymphocytes. It has been shown that cultured macrophages contain reduced levels of the

kinases required to activate dideoxynucleosides such as AZT, and may not be protected by these agents as efficiently as are T cells (100). Thus, it is important to consider the effect of therapeutic strategies for HIV-1 infection in order to address the special biology of HIV-1 infection of the macrophage.

Macrophages are also highly secretory cells. Once activated, macrophages can secrete a variety of materials such as TNF-α, IL-1, and proteolytic enzymes that interfere with cell function and cause cell injury. The inappropriate secretion of these monokines by HIV-1 infected macrophages in brain may induce both neurological symptoms and tissue injury. Macrophages have receptors for, and respond to, several neuropeptides to secrete several toxic oxygen metabolites. These observations provide the basis for a regulatory network in which HIV-1 infected macrophages affect nerve cells through any of several monokines or virus-derived secretory factors. The injured neural tissue in turn reciprocally affects HIV-1 infected macrophages to release more toxic secretory products. Most monokines are autocrine factors that induce other monokines which in turn induce cytokines in lymphocytes, endothelial cells and fibroblasts of adjacent tissue. Macrophages have surface receptors for lymphocytes. During the process of antigen presentation, T cells become activated. One of the consequences is the production of lymphokines, such as GM-CSF. GM-CSF production can then enhance HIV-1 production by macrophages or APC. The virus produced by macrophages can then spread to surrounding $CD4^+$ T cells and ultimately cause the destruction of T cells.

In conclusion, the major reservoir for HIV-1 infection in human peripheral blood is $CD4^+$ T cells. Blood monocytes, in most patients, are not HIV-1 infected. Tissue macrophages are infected and thus are the major reservoir for HIV-1 infection in tissues. When blood monocyte-derived macrophages, from HIV-1 seropositive patients, are placed in culture, they acquire HIV-1 infection from surrounding $CD4^+$ T cells infected with HIV-1. In some patients, monocytes are apparently infected. Further studies are needed to establish how monocytes become infected, if it is a latent or active activation or if it is the result of viral entry with incomplete or partial transcription as reported for resting T cells (54).

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