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**CD4 EXPRESSION ON HUMAN MONOCYTES AND LYMPHOCYTES FROM
HIV SEROPOSITIVE PATIENTS. A FLOW CYTOMETRIC ANALYSIS.**

By

Monika Nowak

THESIS

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

**Department of Physiology
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University of Ottawa**

May, 1991

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ABSTRACT

Mononuclear phagocytes are one of the targets for HIV-1 infection. We wished to study the HIV receptor, CD4, on fresh blood monocytes, as well as a group of cell adhesion molecules (CD11/CD18) on cultured monocytes. The objectives were: (1) to determine the frequency and antigenic density of surface CD4 on blood monocytes, (2) to establish the effect of HIV infection on CD4 density on T cells and monocytes and (3) to determine the effect of growth factors on the expression of CD11/CD18 molecules on cultured monocytes. For the first two objectives, we used a whole-blood, two-color immuno-fluorescence method, a panel of monoclonal antibodies (CD3, CD4, CD8 for T cells and subsets; CD14 for monocytes), and a flow cytometer for immunophenotyping. A quality control program was developed to optimize and monitor the flow cytometer. Various methods of data analysis were used. To investigate the third objective, purified monocytes were cultured with and without GM-CSF, M-CSF and IL-3 and analyzed for the following cell adhesion molecules: CD11a, b, c and CD18 by flow cytometry. We found that all monocytes are CD4+ in normal donors and HIV infected patients. The relative concentration of monocytes was not affected in HIV infected patients in contrast to the decline in T cells. In HIV+ patients, the CD4 density on monocytes is increased ($p < 0.05$) but is decreased on T cells ($p < 0.05$). The lower CD4 antigenic density in T cells correlated with the decreased frequency of CD4+ T cells ($p < 0.03$). A new method of analysis was then implemented to ensure clear separation of these populations. We also observed light scatter abnormalities that together with the abnormal CD4 expression, may cause erroneous data analysis. An increase in expression of all CD11/CD18 molecules was observed with time in culture, and this appeared to be independent of growth factor stimulation. In conclusion, the results suggest that all monocytes are CD4+ and that CD4 antigenic density increases on monocytes but decreases on T cells with HIV infection. We emphasize stringent quality control is required for flow cytometry.

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LIST OF ABBREVIATIONS

AHGG: Heat-aggregated human gamma-globulin
AIDS: Acquired immune deficiency syndrome
APC: Antigen presenting cell
ARC: AIDS-related complex
AZT: Azido-thymidine
CAM: Cell adhesion molecule
CSF: Colony stimulating factor
EDTA: Ethylenediaminetetraacetic acid
FcR: Receptor for the Fc portion of immunoglobulin
FITC: Fluorescein-isothiocyanate
FSC: Forward light-scatter
G-CSF: Granulocyte CSF
GM-CSF: Granulocyte-Macrophage CSF
HIV: Human immunodeficiency virus
IFN: Interferon
IL-1: Interleukin 1
IL-3: Interleukin 3
IL-6: Interleukin 6
kDa: Kilodalton
LPS: Lipopolysaccharide
M-CSF: Macrophage CSF
m.w.: Molecular weight
mAb: Monoclonal antibody
MCN: Mean channel number
MHC: Major histocompatibility complex
nm: nanometer
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PDGF: Platelet derived growth factor
PE: Phycoerythrin
PMA: Phorbol myristate acetate
PMT: Photomultiplier tube
SSC: Side light-scatter
TNF: Tumor necrosis factor

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I dedicate this thesis to the memory of my father.

I. INTRODUCTION

I. 1. PREAMBLE

Analysis of cell surface markers by flow cytometry is a relatively new tool in clinical laboratories. Most methods of analysis were developed in research laboratories and have been particularly useful in the study of leukemias, lymphomas and immunodeficiency diseases (for a review of principles and clinical usefulness see Kern, 1989). Clinical applications have been made possible by the development of monoclonal antibodies directly conjugated with fluorochromes and the development of smaller and simpler air-cooled laser flow cytometers dedicated to cellular analysis without cell sorting capabilities. Unfortunately, flow cytometric analysis of blood samples is not an easy task. It still requires highly trained personnel, well-maintained instruments and experience. Quality control in flow cytometry is of paramount importance for accurate and reproducible measurement of cell surface markers.

The usefulness of flow cytometry has been clearly demonstrated in patients with HIV (Human Immunodeficiency Virus)-1 infection. The cellular receptor for HIV-1 is the CD4 molecule (Barre-Sinoussi et al, 1983) and in this disease, two major target cells, T lymphocytes and monocytes/macrophages bear the CD4 receptor. Measurement of CD4+ T cells has been shown to be the best laboratory method for monitoring progression of the disease and response to therapy (Taylor et al, 1989; Lange et al, 1989; Fahey et al, 1990). CD4 counts are also useful to establish Public Health policy (for example, to estimate the number of patients that will require treatment with AZT or other drugs). The increased usefulness of flow cytometry has uncovered various problems relating to reliability and reproducibility of data and to the assessment of cell surface markers in monocytes, T cells and cells in culture.

In this thesis several issues relating to flow cytometric standardization will be presented, as well as surface CD4 expression and fluorescence intensity by blood monocytes and CD4+ T cells. In the following sections topics connecting the mononuclear phagocyte lineage, flow cytometry and HIV infection will be reviewed succinctly.

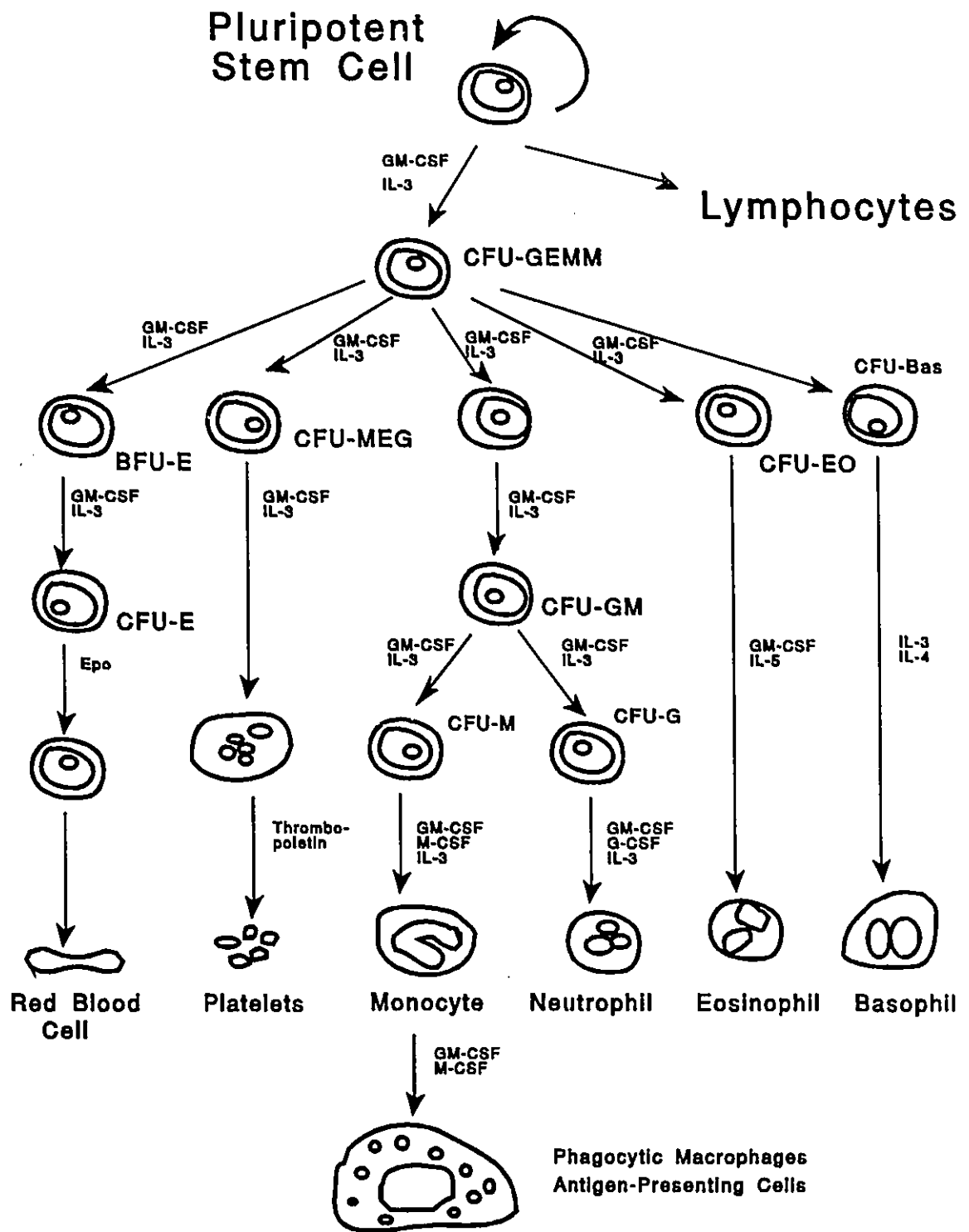
I.2. Mononuclear Phagocyte System

I.2.1. General Physiology

The mononuclear phagocytes represent a cell lineage widely distributed throughout most tissues. The phagocytes play a role in inflammation, host defence, and reaction against a range of autologous and foreign materials

Mononuclear phagocytes originate from a bone marrow precursor which differentiates from a stem cell to form morphologically recognizable monoblasts and promonocytes which then leave the marrow and circulate in the blood for one to three days as blood monocytes (Johnston and Zucker-Franklin, 1988). Upon leaving the circulation and reaching their final abode, the monocytes continue to mature to become "tissue macrophages" (Fig. 1). Depending upon their final destination, the monocytes may mature into alternative end-stage cell types such as Kupffer cells found in the liver, alveolar macrophages in the lung, mesangial cells in the kidney glomerulus, brain microglia, Langerhans cells in the skin, etc. Their turnover in a given tissue under normal conditions has been estimated to vary between 4 and 15 days (Roitt et al, 1989). Macrophages in different tissues have site-specific characteristics. They vary in extent of surface receptors, oxidative metabolism, arachidonate products and expression of Class II Major Histocompatibility (MHC) molecules. Either there are different precursors for each tissue or each tissue microenvironment has a powerful influence on the way the macrophage develops and differentiates.

Figure 1: Cell Lineage Diagram. Lineage diagram of the hemopoietic system showing the actions and interactions of Colony Stimulating Factors (CSF) which promote differentiation and maturation of stem cells and their descendants. Many of the CSFs are pleiotropic and act synergistically with other CSF. CFU-GEMM (Colony Forming Unit-granulocyte-erythrocyte-monocyte-megakaryocyte), CFU-MEG (CFU-megakaryocyte), CFU-EO (CFU-eosinophil), CFU-GM (CFU-granulocyte-macrophage), CFU-E (CFU-erythroid), BFU-E (Burst Forming Unit-erythroid), CFU-M (CFU-macrophage), CFU-G (CFU-granulocyte), CFU-Bas (CFU-basophil), Epo (erythropoietin). For reviews see Metcalf, 1988 and 1989.



With maturation from monocyte to macrophage, the cell enlarges and acquires a large number of lysosomes which are membrane-bound packets containing acid-activated hydrolytic enzymes (Nathan et al, 1980). From the promonocyte to monocyte level, the peroxidase-positive material decreases and the mature macrophage has little peroxidase activity (Nibbering et al, 1987).

In conventional Wright-Giemsa stained preparations, human monocytes appear as large cells (10-18 μ in diameter) with grayish-blue cytoplasm that may contain faint azurophilic granules. The centrally located nucleus is indented or horseshoe-shaped and has a fine, lacy chromatin structure (Fig. 2A and B).

Monocytes may be identified among a variety of hematologic cells by certain distinctive histochemical reactions. The cells possess high levels of alpha-naphthyl butyrase and alpha-naphthyl acid esterase. They are also richly endowed with the lysosomal enzyme acid phosphatase (Yam et al, 1970).

1.2.2. Phagocytic Function

The macrophage response to particulate stimuli is two-fold. First, particles are internalized and subjected to the internal biochemical changes characteristic of endocytosis; second, they synthesize and express bioactive molecules that modulate the response to the environment as well as of the macrophage itself. Through these two basic functions the macrophage is an important component in inflammatory and immunologic reactions.

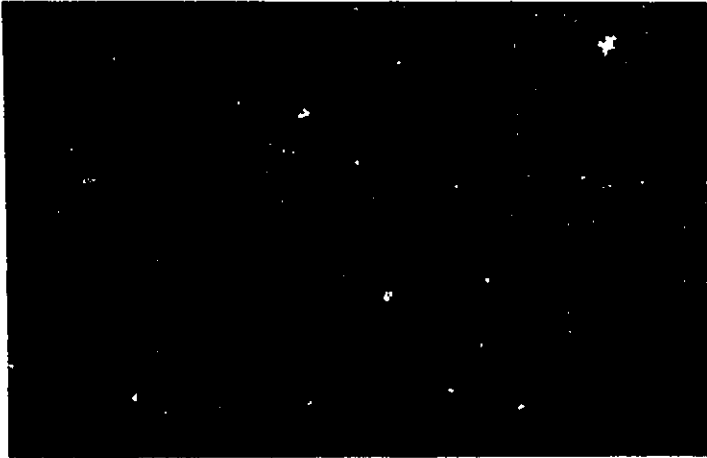
Macrophages are highly active in absorptive endocytosis. Internalization of particulate material results from activation of the actin-myosin contractile system to extend pseudopods around particles. This initiates a respiratory burst in which there is consumption of molecular oxygen and the activation of membrane oxidases that require reduced nicotinamide adenine dinucleotide phosphate. Molecular oxygen will be

Figure 2A and B: Wright-Giemsa Stained Monocytes. Morphological features of peripheral blood monocytes stained with Wright-Giemsa. They average 10-18 μ in diameter and have a horseshoe shaped nucleus.

A



B



reduced to superoxide anion (O_2^-) and eventually to hydrogen peroxide and hydroxyl radical. These oxygen-reactive derivatives have potent anti-microbial and general cytotoxic activity (Nakagawara et al, 1981).

I.2.3. Secretory Function

The macrophage is a highly secretory cell. The variety of secreted molecules is quite extraordinary, ranging from small lipid derivatives such as those from arachidonate to growth-promoting molecules, complement proteins and enzymes. Most of the macrophage secreted molecules are released following their activation. Activation stimuli include primarily bacterial products and lymphokines (for a review see Nathan, 1987). Among the key biological molecules released by macrophages are those that participate in host defence and include IL-1, $TNF\alpha$, $IFN\alpha$ and β (Durum and Oppenheim, 1989).

I.2.4. Immunologic Function

The monocyte is the effector cell of "natural immunity". Natural immunity mechanisms control infection in an antigen-nonspecific manner, generally by phagocytosis of invading pathogens. The monocyte also participates directly in the generation of antigen-specific immune responses by acting as antigen-presenting cells (APCs) (Unanue, 1989).

I.2.5. Role in Inflammation

Macrophages participate in inflammation and in the healing of tissues following injury. They are highly motile cells that respond to chemotactic factors $C5a$, leukotriene

B4 and the formylmethionyl peptides. The role of macrophages in inflammation is complex, involving endocytic function and the release of modulatory molecules that regulate activity of connective tissue cells. Macrophages also secrete various other cytokines (e.g. TNF, PDGF, Transforming Growth Factors α and β).

1.2.6. Role in Infection

In infection, macrophages rapidly mobilize to the infected site, participate as accessory cells for T cell activation (Unanue and Allen, 1987, Weaver and Unanue, 1990) and also rapidly release mediators that result in the acute-phase response. They may be involved in the pathogenesis of atherosclerosis by contributing to the vascular lesion, and insulin-dependent diabetes mellitus, an autoimmune disease where macrophages and lymphocytes infiltrate the islets (Roitt et al, 1989).

The cardinal feature of HIV infection is the depletion of CD4+ T lymphocytes (Fahey et al, 1984; Gottlieb et al, 1981). This selective tropism of HIV for this population of lymphocytes is due to the fact that the CD4 molecule is the receptor for the virus (Barre-Sinoussi et al, 1983; Gallo et al, 1984; Klatzmann et al, 1984; Dalgleish et al, 1984). The viral ligand is the envelope glycoprotein gp120 (McDougal et al, 1986). The consequences of HIV infection are devastating because the CD4 lymphocyte plays a critical role in the immune response. It is responsible directly or indirectly for induction of a wide array of lymphoid and nonlymphoid cell functions. These effects include (1) activation of macrophages (2) induction of cytotoxic T cell, Natural Killer (NK) cell, suppressor T cell and B cell functions and (3) secretion of factors that induce growth and differentiation of lymphoid cells and affect hematopoietic cells. Many CD4+ T cell effects are mediated by the release of a variety of soluble factors that have either tropic or inductive effects on other cell types.

Monocytes/macrophages express the CD4 antigen but the quantity of CD4 antigen expression on the surface is only about one-tenth of that found on the CD4+ lymphocyte (Landay et al, 1990). These small quantities of CD4 antigen on the surface may be sufficient for attachment of HIV-1 but would appear inadequate for extensive viral glycoprotein-mediated cell fusion of CD4+ mononuclear phagocytes. This may not necessarily be true since it has been shown by *in vitro* studies that HIV-1 infected macrophages can form multinucleated giant cells (Lifson et al, 1986). These and other studies also suggest that macrophages could serve as a reservoir for viral persistence and spread (Pauza, 1988; McElrath et al, 1989; Mann et al, 1990; Gendleman et al, 1988; Gartner et al, 1986; Koenig et al, 1986; Nicholson et al, 1986).

Infection by HIV-1 of cells of the mononuclear phagocyte system has been demonstrated in brain (Gendleman et al, 1988; Maddon et al, 1986), lung (Meller et al, 1989; Clarke et al, 1990), lymph nodes (Tenner-Racz et al, 1988; Tenner-Racz et al, 1986), spleen and skin (Braathen et al, 1987). Indeed, reverse transcriptase activity has been detected following cocultivation of monocytes/macrophages from patients with Acquired Immune Deficiency Syndrome (AIDS) and AIDS-related disorders. In addition, both peripheral blood monocytes and tissue macrophages derived from healthy volunteers can support HIV-1 replication *in vitro*.

Recent evidence has suggested that the monocyte/macrophage may play a major role in AIDS as an initial target for infection, as a propagator of virus throughout the immune system (Gartner et al, 1986; Ho et al, 1986; Meltzer et al, 1990) and as a major target cell for infection within the central nervous system (Koenig et al, 1986). HIV infection can remain clinically silent in man for months to years, but with time it can inexplicably lead to profound CD4+ T cell deficiency accompanied by opportunistic infections, neurologic and neoplastic disease, and eventually death (Scadden et al, 1989).

Unlike the CD4+ lymphocyte, the monocyte is relatively refractory to the cytopathic effects of HIV, so that not only can the virus survive in this cell, but also can

be transported to various organs in the body. The pattern of HIV replication in macrophages differs from that of lymphocytes. Macrophages accumulate large numbers of budded virus in intracytoplasmic vacuoles during both acute and chronic infections; release of virus from the plasma membrane is infrequent and at relatively low levels (Gendelman et al, 1988). In contrast, the HIV-infected T cell releases large numbers of viral particles from the plasma membrane (often hundreds of virions/cell section); the number of virions that reside in cytoplasmic vacuoles in these cells is exceedingly small. The noncytopathic-restricted replication of HIV in macrophages is reminiscent of infection with other lentiviruses (e.g. visna virus of sheep) against which effective immune surveillance does not develop. Persistence of HIV in human macrophages may in part explain the inability of an HIV-specific immune response to clear the body of virus. It is interesting to speculate that the macrophage variant of HIV is the form responsible for virus latency and dissemination. Monocyte function in general is abnormal in AIDS or ARC (AIDS Related Complex) patients.

Recent evidence using the cell sorting capabilities of the flow cytometer demonstrate that the primary reservoir of HIV in the peripheral blood is the CD4+ lymphocyte (Schnittman et al, 1989). The polymerase chain reaction, a gene amplification technique, has been utilized to examine highly-enriched sorted monocytes from HIV-1 individuals and has revealed that only a minority of such individuals have demonstrable HIV-1 in their peripheral blood monocytes, whereas all have HIV-1 DNA in their CD4+ T cells. These data raise the question as to what extent cells of the mononuclear phagocyte lineage become infected and where infected cells reside.

The ability to detect HIV infection in the CD4+ T cell fraction correlates with the severity of the disease. Virus production by monocytes is not detectable in the majority of patients and does not correlate with disease severity. McElrath et al (1989) found that the primary source of virus from HIV+ patients' blood is from the CD4+ T cell-enriched fraction. This data does not implicate the monocytes as a primary or exclusive source of

productive HIV infection in any stage of infection. They argue that recent studies suggesting macrophages may serve as a reservoir for virus and as a vehicle for viral dissemination were based upon relatively small clinical samples, and extensive efforts to remove all contaminating T cells were not reported. They suggest caution in attributing monocytes as an endogenous source of HIV infection unless every effort to remove cellular contaminants has been made. Furthermore, the ability to infect blood monocytes *in vitro* does not necessarily correlate with the likelihood that these cells are infected *in vivo* or that they represent a rich source of HIV infection in the human host. Conceivably, the macrophage through its accessory cell function may transfer otherwise nonproductive HIV infection to CD4+ T cells through cell-to-cell contact.

I.2.7. Growth Factors

In general, hemolymphopoietic growth factors appear to act throughout the differentiation cascade of a specific lineage, influencing proliferation and differentiation of stem cells into their progeny, and influencing the functional capacity of the end cells while supporting survival of all classes of cells within a lineage (Metcalf, 1988; Metcalf, 1989). Clear examples of the total lineage effect are seen with M-CSF, GM-CSF, G-CSF and IL-3.

Recent work has also suggested that in general these growth factors tend to act in a stimulatory or inhibitory manner on more than one lineage (Zhou et al, 1988; Johnson et al, 1989). This type of cross-lineage stimulation occurs within the myeloid and lymphoid systems.

It has also been recognized that most growth factors are able to influence the function of mature end cells (Mayer et al, 1987; Coleman et al, 1988). GM-CSF, M-CSF and IL-3 have been found to increase HIV replication in monocytes *in vitro* (Koyanagi et al, 1988). Tables 1-3 list some biological activities of GM-CSF, M-CSF and IL-3.

TABLE I
BIOLOGICAL ACTIONS OF GRANULOCYTE-MACROPHAGE-
COLONY STIMULATING FACTOR (GM-CSF)

I. ACTIVITY ON STEM CELLS:

INDUCES

Colony formation in vitro of:

CFU-GM, CFU-M, CFU-G,

CFU-Meg, BFU-E (with erythropoietin)

II. ACTIVITY ON MATURE END-CELLS

ENHANCES

Cytotoxic and phagocytic activity of neutrophils

Antibody-dependent cell-mediated cytotoxicity

Superoxide anion generation

Survival of neutrophils and eosinophils

INCREASES

Cell-cell adhesion

Expression of cell adhesion molecules in
granulocytes (integrins)

INHIBITS

Neutrophil motility

Tables I-3 were compiled from the following references: Becker et al, 1987, Clark and Kamen, 1987, Sieff, 1987, Geissler et al, 1989, Namiki et al, 1989, Ruef and Coleman, 1990, Breen et al, 1989.

TABLE 2

**BIOLOGICAL ACTIONS OF MACROPHAGE-COLONY
STIMULATING FACTOR (M-CSF)**

I. ACTIVITY ON STEM CELLS**INDUCES**

Colony formation by monocyte/macrophages
precursors: CFU-M

Human M-CSF has low activity on human marrow
but it is very active on mouse marrow

SYNERGIZES

With GM-CSF to increase CFU-M colonies

II. ACTIVITY ON MATURE END CELLS**INDUCES**

Survival of macrophages in vitro

INCREASES

Macrophage antitumor activity
Secretion of O₂ reduction products
Plasminogen activating factor
M-CSF receptor expression

TABLE 3

BIOLOGICAL ACTIONS OF INTERLEUKIN-3 (IL-3)

I. INDUCES

Proliferation of a broad range of hemopoietic
progenitors:

CFU-S, CFU-Blast, CFU-GEMM, CFU-
GM, BFU-E, CFU-G, CFU-M, CFU-EO

Proliferation of leukemic myeloid cells

II. SYNERGIZES

With GM-CSF and M-CSF

1.2.8. Mononuclear Phagocyte Surface Markers

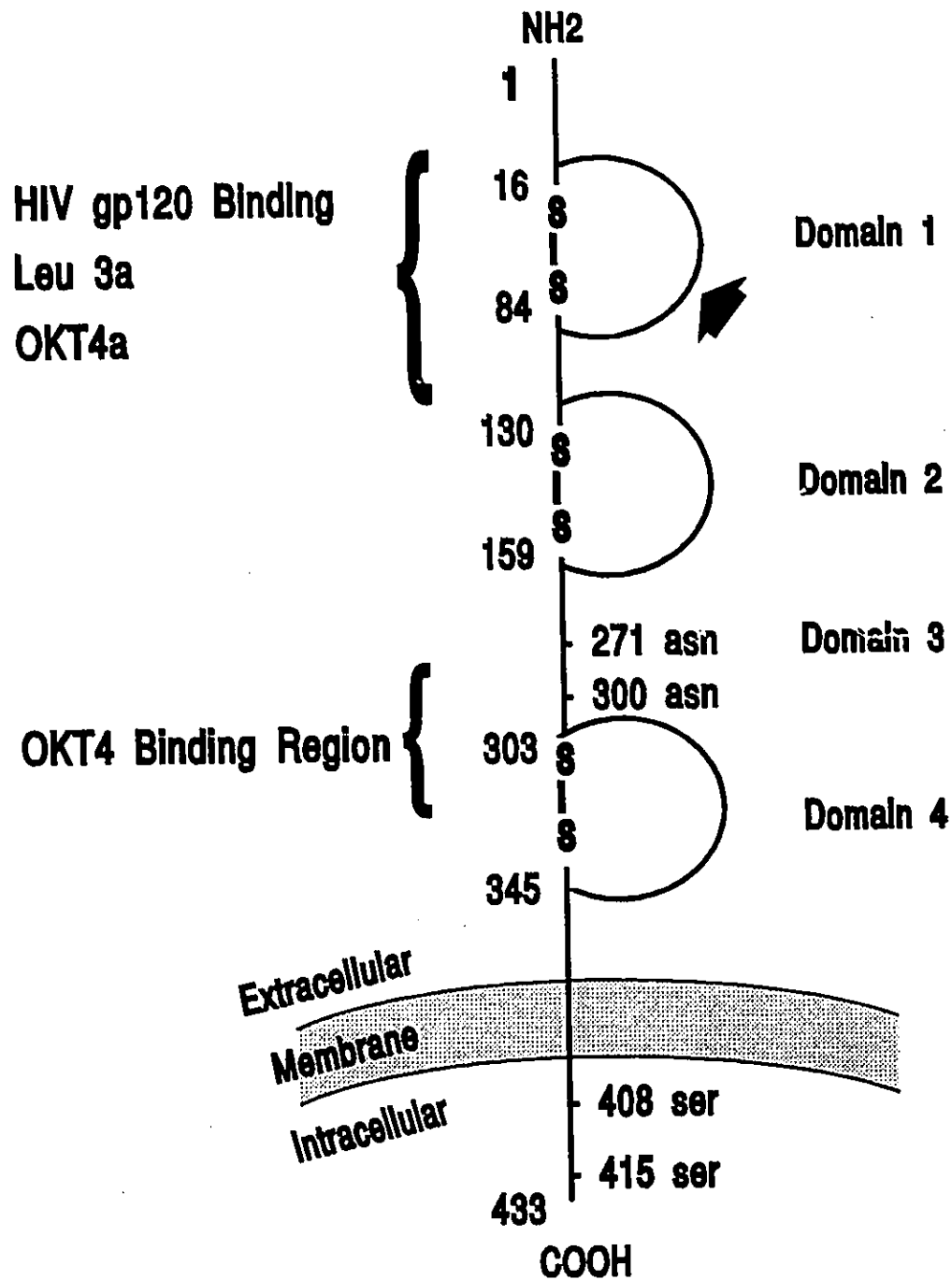
Differentiation of myeloid cells from initial precursor forms to mature, functional cells is accompanied by many changes, including the expression of new proteins on the cell surface. Many monoclonal antibodies have been produced which detect proteins expressed at all or at restricted stages of myeloid differentiation (Todd and Schlossman, 1982). To define these changes at the molecular level and to understand the functions of these proteins, their biochemical characterization and isolation of their genes has begun.

1.2.8.i CD4

CD4 is a 433 amino acid glycoprotein which is in the immunoglobulin supergene family (Fig. 3). It has four extracellular domains, a transmembrane region and a 37 amino acid cytoplasmic portion (Maddon et al, 1987; Landau et al, 1988). The binding of HIV to CD4 is localized to the first two immunoglobulin-like domains ; amino acids 16 to 159 appear to be most important for this interaction and is where anti-CD4 monoclonal antibodies also bind (Landau et al, 1988; Sattentau et al, 1986). Three of the four extracellular domains contain loops formed by disulfide-linked cysteine residues. Asparagine residues at positions 271 and 300 provide glycosylation sites, while serine residues at positions 408 and 415 may be phosphorylated by protein kinase C.

The infectious route of entry of HIV as shown biochemically and by electron microscopy involves : 1. binding/fusion of gp120 to CD4 surface receptor; 2. internalization of receptor-virus complex (or just virus); 3. fusion between viral and endosomal membranes with release of nucleocapsid into cytoplasm (Clapham and Dalgleish, 1988). In monocytes, internalization and degradation of HIV is very rapid (helped by phagocytosis) and has been demonstrated to reside within endosome-like vesicles (Gendelman et al, 1988).

Figure 3: CD4 Molecule. Diagram of the structure of the CD4 glycoprotein. CD4 is comprised of 433 amino acids and has four extracellular domains, three of which contain immunoglobulin-like loops formed by disulfide-linked cysteine residues. Asparagine residues at positions 271 and 300 provide glycosylation sites, while serine residues at positions 408 and 415 may be phosphorylated by protein kinase C. The region from amino acids 16-159 forms the main binding sites for HIV gp120 and for OKT4a, Leu3a monoclonal antibodies. The arrow indicates the HIV binding region.



1.2.8.ii CD14

Differentiation of myelomonocytic cells from pluripotent stem cells to mature, functioning monocytes/macrophages is accompanied by increased expression of the CD14 cell surface antigen. CD14 is a 55 kDa (kiloDalton) glycoprotein joined to the plasma membrane by a phosphatidylinositol glycan anchor (Simmons et al, 1989). Treatment of macrophages with phosphatidylinositol-specific phospholipase C (PI-PLC) results in the removal of 70% of cell surface CD14 (Haziot et al, 1988). It is a well-known marker for monocytes and macrophages and is recognized by several monoclonal antibodies commonly used in flow cytometry studies (LeuM3, Mo2 and MY4).

The genes for both human and murine CD14 have been cloned and sequenced. It is composed of 365 amino acids and appears to have cell surface and soluble forms which have been detected in plasma and urine. The molecular weights of both appear identical, which argues against existence of distinct secreted and integral membrane-bound forms differing by the presence or absence of a hydrophobic transmembrane segment. It may be a peripheral, easily-shed glycoprotein (perhaps by an endogenous monocyte-specific phospholipase).

The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors (chromosome 5-q arm) and its deletion in the malignant cells of patients having myeloid leukemias suggest that the CD14 antigen may serve as some type of receptor and that a role for this gene in the pathogenicity of myeloid disorders should be considered (Goyert et al, 1988). However, according to Simmons et al (1989), patients with paroxysmal nocturnal hemoglobinuria (PNH), an acquired defect of hematopoietic cells which leads to a selective absence of glycosyl phosphatidylinositol (GPI)-linked carboxy termini, had normal levels of mature monocytes, greater than 95% of which lacked detectable CD14 expression. This argues that CD14 probably is not a growth factor or receptor essential for monocyte maturation. In addition, monocytes stripped of

CD14 by monoclonal antibody modulation show no defect in antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis of immunoglobulin-coated erythrocytes.

A biological function has only recently been ascribed to this molecule. Wright et al (1990) have shown that CD14 binds Lipopolysaccharide Binding Protein (LBP)-Lipopolysaccharide (LPS) complexes and thereby serves two important functions. LBP (a serum protein that binds LPS) acts as an opsonin, so CD14 may thus function in the clearance of Gram-negative pathogens during infection. LBP also promotes the secretory responses of cells to LPS, and CD14 may thus function to heighten the sensitivity of the immune system to infection.

1.2.8.iii Cell Adhesion Molecules

Physical communication among cells of similar or different lineages is mediated by groups of cell-surface molecules collectively known as Cell Adhesion Molecules (CAMs). CAMs play an important role in embryogenesis, immunological functions, hemopoiesis etc. CAMs mediate many leukocyte functions like leukocyte adherence, transendothelial migration, infiltration of inflamed tissues and adherence to endothelial cells (Kishimoto et al, 1987; Klingemann and Dedbar, 1989).

Leukocyte CAMs are also known as Leukocyte Integrins and are differentially expressed among blood cell lineages. The integrins are a superfamily of heterodimeric adhesion molecules consisting of noncovalently associated α and β chains (Fig. 4). The superfamily is presently divided into three groups based on the sharing of a common β chain (i.e. β 1, β 2 or β 3). The leukocyte integrins (the β 2 family) are LFA-1 (renamed CD11a/CD18), CR3 or Mac-1 (CD11b/CD18) and p150,95 (CD11c/Cd18) which have α chains of 175, 165 and 150 kDa respectively (Table 4) (Sanchez-Madrid et al, 1983). The β chain (CD18), 95kDa, is required for these heterodimers to be expressed on the membrane surface (Kishimoto et al, 1989). CD11a and CD18 are synthesized from

Figure 4: The Integrin Supergene Family. Overview of the structure of the Integrin supergene family. The Integrin family includes at least 10 different, but structurally related, cell surface heterodimers composed of noncovalently linked α and β chains which are involved in both the attachment of cells to extracellular matrix (ECM) proteins as well as in cell-cell interactions. Three distinct subfamilies of Integrins have been identified based on the presence of a common β chain which is associated with a distinct α chain. The subfamilies that use the β_1 and β_3 chains function primarily as ECM protein receptors, while the subfamily that uses the β_2 chain includes the leukocyte adhesion proteins whose major function is to strengthen cell-cell interaction.

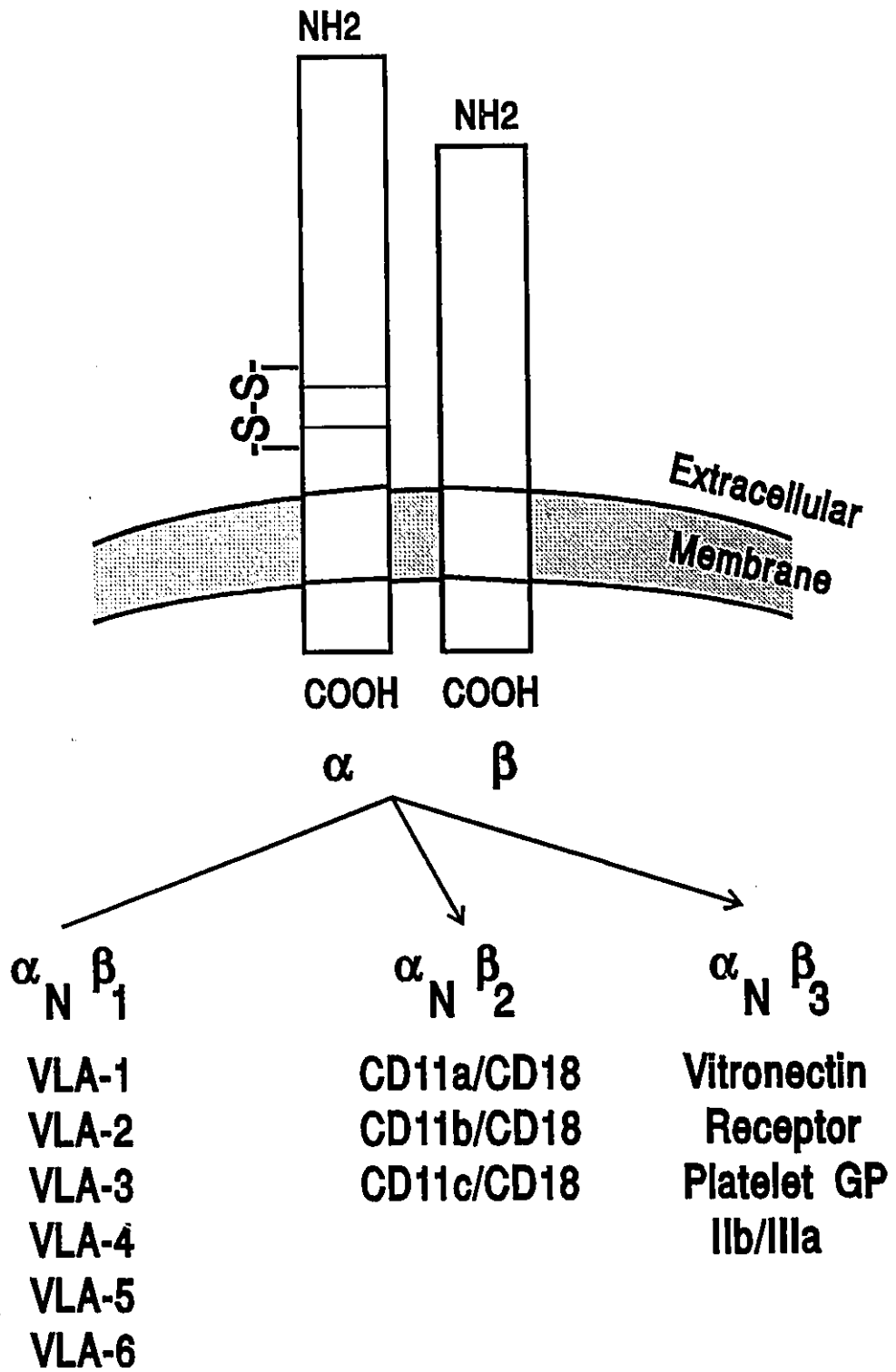


TABLE 4

**LEUKOCYTE ADHESION MOLECULES CD11/CD18
(β_2 INTEGRINS)**

MOLECULE	m.w. (kDa)	ANTIBODY	CELLULAR DISTRIBUTION	LIGAND
CD11a	$\alpha=180$ $\beta=95$	anti-LFA-1 (α chain)	T, B, NK, M, N	CD54 ICAM-2
CD11b	$\alpha=170$ $\beta=95$	anti-C3biR	M, N, some T, NK	C3biR
CD11c	$\alpha=150$ $\beta=95$	LeuM5 (B-D)	M, N, some T	? iC3b
CD18	95	anti-LFA-1 β chain	T, B, NK, M, N	

Tissue distribution of the β_2 Family of Leukocyte Integrins: CD11a/CD18, CD11b/CD18, CD11c/CD18 and the common β chain CD18. CD=Cluster of Differentiation, the nomenclature adopted by the International Workshop on Human Leukocyte Differentiation Antigens. m.w.: molecular weight, kDa: kiloDalton, T:T cell, B:B cell, M: monocyte, N:neutrophil, NK:Natural Killer, B-D: Becton Dickinson.

independent precursors. This group of glycoproteins mediate cellular adhesion. Leukocyte Adhesion Deficiency (LAD) is a recently recognized autosomal-recessive trait characterized by recurrent bacterial infections, impaired pus formation and wound healing, and abnormalities in a wide spectrum of adherence-dependent functions (e.g. failure to accumulate at inflammatory sites) of granulocytes, monocytes, and lymphoid cells (Todd and Freyer, 1988; Springer et al, 1984; Anderson and Springer, 1987). Features of this disease are attributable to the deficiency (or absence) of cell surface expression of the family of functionally and structurally related glycoproteins outlined above. Recognition of the molecular pathogenicity of this disorder has allowed rich insights into the role of cellular adherence reactions in inflammatory and host defence.

CD11a predominates on lymphocytes but is present on all leukocytes to some degree; it is known to be the receptor for ICAM-1 (the rhinovirus receptor) expressed on non-hemopoietic cells (Marlin and Springer, 1987). Recently Valentin et al (1990) discovered that CD11a/CD18 participates in HIV induced syncytium formation by lymphoid cells. When anti-CD11a was used in humans, it prevented graft failure in HLA-mismatched bone-marrow transplantation and hence may have a therapeutic potential.

CD11b predominates on granulocytes (i.e. neutrophils) and is the receptor for the complement fragment C3bi and is possibly the receptor for fibrinogen and Factor X. As mentioned, CD11b/CD18 is essential for binding to C3bi and other ligands, spreading homotypic adhesion, phagocytosis of opsonized particles in neutrophils (a role also subserved by CD11c/CD18 in resident macrophages). Granulocytes and monocytes have intracellular storage pools of CD11b and CD11c, while tissue macrophages do not contain intracellular pools of these receptors (Todd et al, 1984). In dogs anti-CD11b/CD18 reduced experimental myocardial injury and size of infarct and the accumulation of granulocytes was attenuated.

CD11c/CD18, a recently characterized member of the CD11/CD18 complex, is

found on neutrophils and monocytes and may contribute to the adherence of these cells to other cells. In fact, CD11c/CD18 promotes adhesion-dependent phenomena such as adherence to endothelial cell monolayers. CD11c predominates on macrophages and is also believed to bind the C3bi fragment of complement (Lanier et al, 1985). It is also a marker for Hairy Cell Leukemia on B cells (Schwartz et al, 1985).

Monocytes and neutrophils may depend upon the enhanced expression of CD11/CD18 glycoproteins to facilitate contact with vascular endothelium thus allowing emigration and diapedesis to inflammatory foci within affected tissue compartments (Luscinskas et al, 1989).

Exposing neutrophils to the Ca⁺⁺ ionophore A23187 or fMLP or PMA caused an increase in CD11b/CD18 and CD11c/CD18, while CD11a/CD18 was unaffected (Freyer et al, 1988). It was also determined that monocytes in a mononuclear fraction also responded to the soluble stimuli C5a and fMLP with a similar increase in CD11b/CD18 and CD11c/CD18, but with A23187 an increase was observed in the level of all three markers on monocytes (the change was temperature-dependent). By stimulating various tissue macrophages with A23187 stimulus, it was found that pulmonary alveolar macrophages (PAM) do not exhibit significant alteration in surface antigen density and that the baseline antigen expression by macrophages is heterogeneous, from negligible to barely detectable CD11a,b,c/CD18 expression. Variable expression of breast milk macrophages (BMM) and both PAM and BMM did not respond to A23187 stimulus.

It can be speculated that since tissue macrophages have already breached the endothelial barrier during their ontogeny, they therefore may have no need to express and/or regulate the surface density of the CD11/CD18 class of adhesion-promoting molecules.

The mechanism by which CD11/CD18 enhances cell adhesion is not well understood. Data suggest a qualitative change in CD11/CD18-mediated function: a quantitative rise in CD11a/CD18 does not occur acutely and is not required for early T

and B cell adhesion responses. Also, several CD11b/CD18 mediated functions occur despite no measurable increase in surface expression of this receptor, and certain functions of CD11/CD18 (e.g. neutrophil aggregation) are usually reversible despite a continued increase in surface expression of this receptor .

The identification of the CD11/CD18 glycoprotein complex has led to an investigation of cellular mechanisms that may modulate the expression of these adhesion-promoting glycoproteins on the plasma membrane of human leukocytes. These glycoproteins expressed by circulating monocytes may be under regulatory control but little is known about their expression outside tissue macrophages which may rely on these surface glycoproteins to facilitate adhesion-dependent interaction.

I.3. FLOW CYTOMETRY

I.3.1. Evolution of Surface Marker Assays

Surface marker analysis is a relatively new laboratory tool, which has been particularly useful in studying leukemias, lymphomas, and immunodeficiency diseases. In the past few years, the diagnostic use of surface marker analysis was greatly expanded with the development of monoclonal antibodies as discrete probes and flow cytometry for objective quantification of large numbers of cells (Parks et al, 1986). Traditionally, morphologic characteristics have been used to classify cells by groups with functional similarities. Unfortunately, morphology alone has proved inadequate in distinguishing the many functional capabilities of lymphocyte subsets (T cells, B cells, Natural Killer cells, etc). In immunodeficiency disease, subsets of lymphoid cells can provide important information about classification of disease, prognosis, and genetic counselling. To distinguish between lymphocytes with various functions, techniques including analysis by rosetting, immunofluorescence microscopy, enzyme histochemistry, and most recently, flow cytometry with monoclonal antibodies have been developed.

As the term implies, flow cytometry is the measurement (-metry) of cellular (cyto-) properties as they move in a fluid past a stationary set of detectors. The flow cytometer is a result of the blending of computer science, electronics, hydrodynamic focusing and ink-jet technology, optics and light detection, and monoclonal antibodies. The result is an instrument capable of rapid, multiparameter analysis of heterogeneous cell populations on a single-cell analysis.

1.3.2. History of Flow Cytometry

As Howard Shapiro (1988) has documented, quantitative cytology and, therefore, flow cytometry, had its beginnings in the work of Caspersson and his colleagues in Stockholm in the 1930's to the early 1950's (Caspersson and Schultz, 1938; Caspersson, 1950). Their work and the work of others pioneered the use of absorption measurements for the study of individual cellular components. During this time, Papanicolaou and Traut (1941) demonstrated the clinical utility of nuclear chemistry morphology in the diagnosis of malignancy in cytological specimens. In the same era, Coons and Kaplan (1950) demonstrated the first use of immunofluorescence multiparameter flow cytometers that could rapidly measure multiple innate cellular parameters, measure multiple wavelengths of fluorescence emission, and physically sort cells.

In the middle 1960's Kametsky and Melamed (1967) built a multiparameter instrument to discriminate normal from malignant cervical cells. This instrument utilized absorption at 260nm as a measurement of nucleic acids, and light-scatter at 410nm as an indicator of cell size. At the same time, Fulwyler and colleagues at Los Alamos National Laboratory produced an instrument that used the Coulter principle (Coulter, 1956) of electronic cell volume combined with the newly introduced ink-jet printer technology to physically sort homogeneous cell subpopulations from the heterogeneous cells of peripheral blood (Fulwyler, 1965).

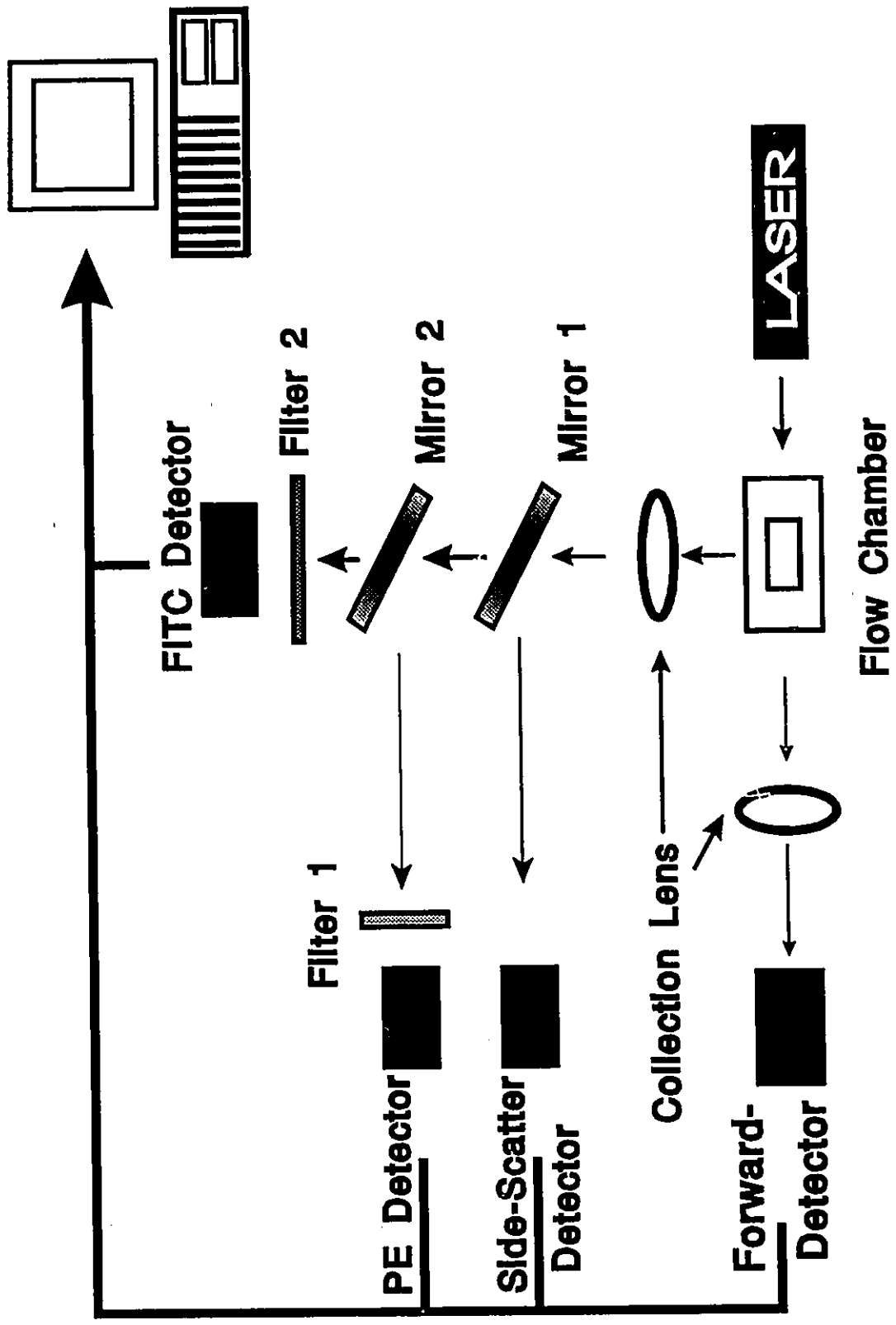
1.3.3. Fundamentals of Flow Cytometry Instrumentation

In the simplest terms, a flow cytometer operates by causing cells in a fluid stream to pass single file through a beam of light, usually generated by a laser. The photons of light that are scattered and emitted by the cells following their interaction with the laser beam are separated into constituent wavelengths by a series of filters and mirrors (Fig. 5). This separated light falls on individual detectors that generate electrical impulses, or analog signals, proportional to the amount of incident light striking the detectors. Each analog signal is converted to a digital signal. These individual numbers are accumulated in a frequency distribution, or histogram. The numbers are proportional to the amount of light emitted from, or scattered by, individual cells.

To perform these functions, the cytometer needs a combined system of (1) Fluidics, to introduce and restrict the cells for interrogation (hydrodynamic focusing), (2) Optics, an excitation source (laser) and collection optics (filters) to generate and collect the light signals and (3) Electronics, to convert the optical signals to proportional electronic signals (voltage pulses) and digitize them for computer analysis and display. Additional systems are included in instruments capable of preparative cell sorting.

The specimen must be presented to the instrument in suspension. In order to analyze the cells, one must establish and maintain a highly controlled fluid stream designed to provide the exact location of the specimen in 3 dimensions. This is achieved in the sample-handling compartment by forcing a usually isotonic particle-free fluid (called a sheath fluid) under pressure through a conical nozzle assembly geometrically designed to produce a laminar flow. The nozzle has an orifice 50μ to 250μ in diameter through which the fluid exits at a high flow rate, typically 10 m/sec. The sample, in its own isotonic fluid, is introduced at a higher differential pressure than the sheath stream into the nozzle through an insertion rod. The laminar flow induced by the nozzle imparts a hydrodynamic focusing effect that locates the sample stream in the center of the sheath

Figure 5: Diagram of a Single-Laser Flow Cytometer. A generalized flow cytometer system. Flow cytometers make measurements based on light (laser) as the source of excitation. The scattered and fluorescent light generated by cells passing through the illuminating beam is collected by photodetectors which convert the photon pulses into electronic signals. Further electronic and computational processing results in graphic display and statistical analysis of the measurements being made.



stream. Thus, a coaxial stream within a stream is created; the sheath fluid forms around the sample fluid. Under these conditions, with optimized differential pressure, the sample stream is no wider than the cells it contains.

All commercial flow cytometers use a light source (usually one or more lasers) for the excitation of fluorochromes and quantification of intrinsic cellular parameters. Laser light is coherent; all the waves of light are parallel and it is monochromatic (of a single wavelength or frequency, representing a single color). New flow cytometers have efficient optical systems and operate with lower power lasers that may be air-cooled instead of water-cooled.

Emitted laser light is passed through beam-shaping optics that shape and concentrate the light to a fine spot. The point at which the laser beam and the cell stream meet is called the interrogation point. Alignment is critical to successful operation of flow cytometers; suboptimal alignment can result in erroneous data collection, presentation, and hence interpretation.

I.3.4. Optical Analysis

1.3.4.i Light-Scatter Analysis

Once a cell has reached the point in the sample stream that intersects with the light beam, two events will occur, assuming that fluorochromes are in or on the cell. The first event is that the cell will scatter light from the beam at the incident wavelength through 360°. The dynamics of light scatter is a complicated physics problem involving diffraction and reflection of light by the cell. Briefly, if one collects light scattered along the axis of the laser beam (a parameter known as forward-angle light-scatter), the quantity of light is proportional to the size of the particle or cell. If the scattered light is collected orthogonally at right angles to the laser beam, the parameter is termed 90° light scatter, or side-scatter. Empirically, 90° scatter has been shown to be composed primarily

of light reflected by internal structures or membrane undulations. Therefore, this parameter correlates with cell granularity. The properties of forward and 90° light scatter are termed intrinsic properties because they can be measured by the instrument without the aid of exogenous reagents.

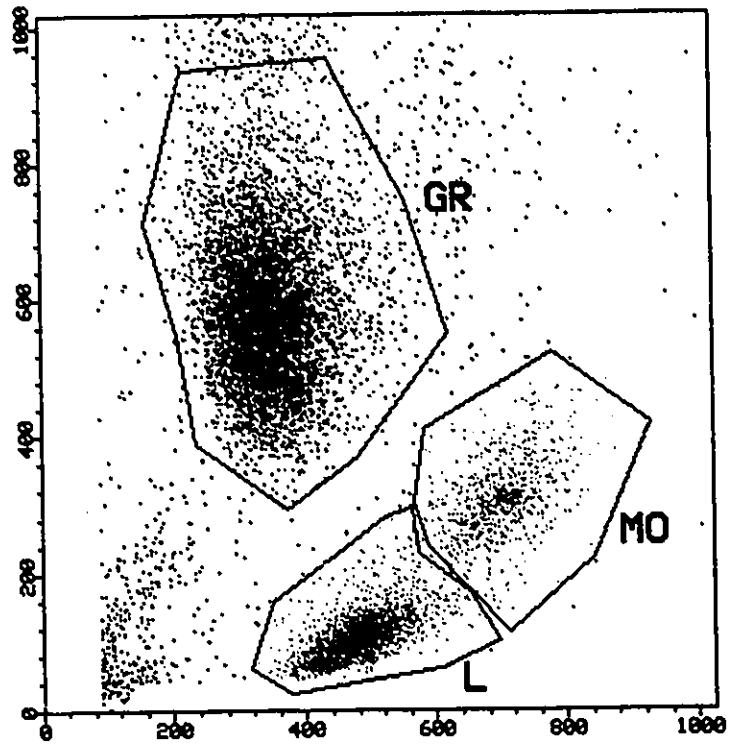
As an example of intrinsic properties, if one were to analyze a peripheral blood specimen from which the red blood cells have been removed, one would find that lymphocytes, relatively small cells with monotonous cytoplasm and regular nuclei, exhibit both low forward-angle scatter and low 90° scatter (Fig. 6). Since granulocytes are larger cells with multilobed nuclei and many cytoplasmic inclusions, they have increased scatter properties. Monocytes show intermediate forward and 90° scatter patterns. The sensitivity of light-scatter measurement is such that different cell populations may all be readily distinguished from one another.

1.3.4.ii Fluorescence Analysis

The second event that occurs at the laser interrogation point is that fluorochromes present on or in the cell absorb the laser light and re-emit the light at a lower energy and longer wavelength. This is the property known as fluorescence. Each fluorochrome possesses a distinctive spectral pattern of absorption (excitation) and emission. Typically, with argon-ion lasers, the excitation wavelength used is 488nm, a blue to blue-green light. The fluorochrome must also emit light at a wavelength sufficiently longer than the excitation wavelength so that the 2 colors of light may be optically separated with selective filters. The most popular fluorochrome used in immunofluorescence analysis is fluorescein-isothiocyanate (FITC), which has an absorption maximum between 450nm and 540nm. The difference between the absorption and emission wavelengths is known as Stokes' shift. With all fluorochromes, a wider Stokes' shift is more useful to the flow cytometrist. If multiple fluorochromes are used, their emission

Figure 6: Light-Scatter Diagram of Whole Blood Leukocytes. A light-scatter diagram (cytogram or dot plot) of a sample of human peripheral blood leucocytes. Light scatter collected at right angles to the laser beam (termed side-scatter) measures the parameter that correlates with cell granularity. Light scatter collected at forward angle (0-10°) scatter is proportional to cell size. The lymphocytes are relatively small cells with agranular cytoplasm and regular nuclei and hence exhibit both low forward and side scatter patterns. Since granulocytes are larger and have multilobed nuclei and many cytoplasmic inclusions, they have increased scatter properties. Monocytes show intermediate forward and side scatter patterns. A "gate" has been set around each population. L (Lymphocyte cluster), Mo (Monocyte cluster), Gr (granulocyte cluster)

**Forward Scatter
(Cell Size)**



**Side Scatter
(Granularity)**

spectra must have minimal overlap so as to be separately quantified; but ideally, they should have the same or similar absorption spectra to allow excitation with a single wavelength of light and a single laser.

The light produced in the fluorescence event, like the scattered light, is emitted through 360° but is collected by a lens at 90° to the jet and laser-beam directions. Therefore, multicolored light enters the detection chamber (the scattered light at the incident wavelength, and the fluorescent light at longer wavelengths). The amount of light (or number of photons) is proportional to the amount of fluorochrome present in or on the cell. The multicolored light is separated into its constituent wavelengths (colors) by dichroic reflector filters and each is directed to a different detector. Separation is accomplished with short- and long-pass filters which are interference or absorption filters. Dichroic mirrors reflect the light not allowed to pass. In this manner dichroic mirrors may be used to send light of one color to one detector and light of another to a second detector. Thus, light from each fluorochrome may be physically separated and individually quantified.

1.3.5. Data Collection

The photons of light impinging upon either the scatter or fluorescent detectors are converted to electrical impulses proportional in magnitude to the number of photons received. Generally, these impulses are analog signals varying in magnitude from 1 to 10 volts. In addition to the modes of signal processing, the signals may be collected in either linear or log domain. Collection of logarithmic signals affords a broader range of fluorescence detection and more sensitivity in the low fluorescent range.

The principle method of collecting digitized data is in list-mode. List-mode analysis requires that the operator select which parameters are to be observed. As each cell is interrogated, the digitized data from each and every parameter is stored in the

computer as a "list". This list is a matrix containing all of the collected data on all of the analyzed cells and may subsequently be processed by the computer to form histograms.

In addition to multiparameter analysis, the concept of electronic "gating" is important to an appreciation of flow cytometry. A gate may be defined as an electronic window encompassing a given region of a distribution, set off by upper and lower limits and used to set off a subpopulation from a heterogeneous distribution. The instrument may then be instructed to form a histogram of the fluorescence distribution of only the gated events while excluding the others. This is one of the most powerful aspects of flow cytometry and may be used to ask very specific questions about a well-defined subpopulation of cells.

1.3.6. Data Display and Evaluation

Several forms of data display and numerical evaluation are commonly employed in analyzing flow cytometry data. These include single-parameter displays (e.g., histograms), contour plots and similar displays that show cell frequency distributions as a function of paired measurements on two instrument sensors, and sample/subsample statistics such as the percentage of cells falling within specified ranges of sensor reading and means or medians of signal levels.

1.3.7. Single-Parameter Displays (Histograms)

The simplest way to display one-parameter digital flow cytometry data is to plot the number of cells with a particular signal level as a function of the signal level. For example, with linear data, the x-axis is divided into 256 brightness ranges (channels) and the number or frequency of cells falling in each channel is plotted in the y-direction. The area under the line is proportional to the total frequency of cells in that signal-level

region, making it possible to visually estimate the relative frequencies of two populations.

1.3.8. Two-Parameter Displays: Dot Displays and Contour Plots

Typical two-parameter flow cytometry data contain much more information than can be shown on two single-parameter histograms. A full two-parameter display has a place for cells with any combination of the two measurement values and a way to indicate the frequency of cells in any region.

Dot displays are the simplest two-parameter displays to construct: a two-dimensional region is defined in which the x-dimension represents the signal for one parameter and the y-dimension represents the signal for the other; a dot is placed at the x-y point that corresponds to the measurements for each cell in the data. The frequency in any region is represented simply by the number of dots in the region. Some flow cytometry software provides "quadrant statistics" (termed Quadstat) in which a pair of lines, one vertical and one horizontal, are used to divide a two-parameter display into four regions (quadrants) and the fraction of cells in each region is calculated. This is quick and convenient for preliminary analysis.

Contour plots are derived in effect by converting a three-dimensional surface to a two-dimensional form that can be displayed on a screen and printed. The three-dimensional surface is marked with "contour" lines that indicate where the surface crosses specified cell frequency levels. In its simplest form, where the contours are drawn at fixed intervals, this display is much like a geologic survey topographic map where the lines represent land surface elevation.

1.3.9. Sample and Subsample Statistics

In flow cytometry, the usual objective in calculating sample statistics is to condense selected characteristics of a cell population (such as its relative frequency, typical signal level, and the shape of the distribution) into a few numbers to facilitate tabulation and comparison of results.

Evaluating the fraction of cells in a subpopulation is easy if the subpopulation is well-defined by the data, but difficulties occur when positive staining and control (negative) populations are not well-resolved. If such an unresolved signal distribution is composed of two subpopulations, that is, some of the cells are positive for the labelled characteristic and some are negative, any cutoff selected to define positive cells will underestimate their true frequency. In cases where one believes that all the cells are part of a single population but there is overlap between stained and control signal distributions (e.g., when a clonal population is stained for a low-density cell-surface molecule) stating a percentage of positive cells is inappropriate, and an estimate of the typical signal levels for stained and control populations is more reasonable.

With the proper array of light sources, detectors, and filters, multicolor (i.e. multiple-marker) analysis is straightforward. The two major pitfalls in performing these assays are (1) overlap of the emission spectra of the fluorochromes, and (2) hindrance (steric or competitive) of the binding of multiple monoclonal antibodies to the cells. The overlap of emission spectra results in the signal from one fluorochrome "bleeding" over into the detector for a second fluorochrome, because some of the signal is of the same wavelength and cannot be removed by the optical filters. This is overcome in 2 fashions: (1) fluorochromes that are used together are chosen for their distinct and tight emission spectra peaks, and (2) the effect of the residual "bleed-over" is minimized by a procedure termed fluorescence (electronic) compensation (also termed subtraction). Fluorescence which cannot be optically filtered.

1.3.10. Quality Control

As with other experimental assays, analysis and interpretation of data accumulated by flow cytometry cannot be attempted unless proper attention is directed toward controls and calibration. The general areas of concern with assays requiring flow cytometric analysis are (1) proper and reproducible alignment and calibration of the cytometer, (2) historical databases demonstrating the distribution of the marker on normal specimens, (3) positive and negative controls performed concomitantly with the clinical test to ensure that the assay is working satisfactorily on a given day, and (4) proper specimen handling and sample preparation.

The alignment and calibration of a flow cytometer is usually accomplished with stable, fluorochrome-conjugated fluorescent beads. This is performed to ensure the instrument is properly configured for the desired analysis, and to minimize the day-to-day variability in analyses. Thus, beads with constant amounts of fluorescence are run daily, the laser power and/or high-voltage settings for the photomultiplier tubes (PMTs) are adjusted to bring the fluorescence peak of the particles to a consistent channel, and the settings are recorded in a log. This reduces the possibility that differences in the staining of cells or markers are due to changes in the ability of the cytometer to detect fluorescence in a consistent manner.

A historical database is important because it defines the distribution of a marker or markers on normal populations of cells, given a consistent alignment and calibration of the cytometer. Negative controls consist of staining cells with nonspecific antibodies of the same isotype as the antibodies directed against the surface marker. This indicates the amount of nonspecific binding unrelated to the presence of antigen on the cell surface. The staining technique (either direct or indirect) used with the isotype control should be the same as that used with the specific antibody.

1.3.11. Application of Flow Cytometry to the Study of HIV Infection

The individual course of disease progression between HIV infection and development of AIDS is highly variable. Flow cytometric evaluation using monoclonal antibodies (mAb) against cell surface differentiation antigens provides a powerful tool to assess the extent of immunologic damage and predict survival in individual patients. A classification scheme based on non-random development of immunologic abnormalities assessed by flow cytometry is useful in staging both symptomatic and asymptomatic HIV-infected individuals (Zolla-Pazner, 1987). In addition, flow-cytometric information can be used to determine whether treatment with antiviral drugs is appropriate or has been effective in arresting immunologic deterioration. CD4+ and CD8+ T cell levels are very informative lymphocyte subset parameters for following disease progression in HIV infection (Taylor et al, 1989).

Four stages of CD4+ cell loss occur during HIV disease (Giorgi et al, 1987). In the first stage, during the first 12-18 months after seroconversion, the mean CD4+ cell level falls from $1000/\text{mm}^3$ to $600/\text{mm}^3$. During the second stage, which can last several years, CD4 levels are relatively stable, although some studies have demonstrated a small but steady fall in CD4+ cell number during the asymptomatic period. Further rapid CD4+ cell loss leads to the development of AIDS and characterizes the third stage. In the fourth stage, from AIDS diagnosis to death, CD4+ cell levels fall even further. CD4+ cell decline probably results at least in part from failure to suppress *in vivo* HIV replication. CD4+ cell levels are highly prognostic for predicting survival, especially during the last two stages, and death occurs within 1 year in about 80% of untreated people whose level has fallen to 10% CD4+ cells. CD4+ cell percentages are slightly more predictive for AIDS free survival than CD4+ cell absolute counts (Landay et al, 1990). Throughout the course of HIV disease, the total T cell levels remain fairly constant in spite of the fall in the CD4+ cell number. This is due to the concomitant CD8+ lymphocytosis.

The sum of CD4 and CD8 should approach the number of CD3+ cells within $\pm 10\%$ (Landay et al, 1990). NK cells express CD8 and if a person has many CD8+ NK cells, their CD4+ plus CD8+ values may be greater than the CD3 by $> 10\%$. Second, if monocytes (which are dimly CD4+) are included in the gate, they will give exaggerated CD4+ values. The mean CD3+ cells (total T cells) should approach 70-75% of lymphocytes since the CD3+percentage remains fairly constant throughout HIV disease.

TABLE 5
Reference Values for Lymphocyte Subsets

Lymphocyte subset	Median ($\pm 10\%$)	
	%	Absolute Counts*
Total T cell	72	1214
T _{helper/inducer}	45	739
T _{suppressor/cytotoxic}	26	428
Total B cell	10	169
Total NK** cell	13	228
Ratio (CD4/CD8)	1.74	

* absolute number of cells/mm³ ; **NK, natural killer cells

The values in Table 5 were obtained from healthy laboratory HIV-seronegative volunteers, expressed as percentage of total from flow cytometric analysis (Landay et al, 1990).

1.4. RATIONALE

The introduction of flow cytometry to clinical laboratories has had a great influence on the use of cell-surface markers as a means to evaluate lymphocyte subsets in health and disease. Perhaps the best example is in the role that CD4+ T cell counts have in the management of patients and clinical trials of new therapies against HIV infection.

In HIV infection, there are two major circulating target cells carrying the receptor for HIV (the CD4 molecule), a T cell subset and monocytes. HIV infection is characterized by a steady and slow decline in CD4+ T cells in peripheral blood. Studies (Fahey et al, 1990) have shown that measurement of CD4+ T cells is the best laboratory method for monitoring disease progression and response to therapy. In clinical trials, the value of the CD4+ T cell count is the main laboratory criterion for entry to the trial, for example, only patients with < 500 CD4+ T cells/ml may be admitted to the trial.

It is well-known that blood monocytes are CD4+, however, the exact percentage of monocytes that are CD4+ is not known. Various published papers (Crowe et al, 1987; Szabo and Micbr, 1990; Lucey et al, 1991) report from 20 to 75% of monocytes are CD4+. It has also been reported that the fluorescence intensity (antigenic density) decreases in T cell lines infected with HIV-1 (Crise et al, 1990; Stevenson et al, 1987; Hoxie et al, 1986). Both observations remain highly controversial and the major aim of this thesis is to resolve these questions.

The increasing use of flow cytometry has uncovered several areas that require further development. Perhaps the most important areas are those dealing with issues relating to the instrument, antibodies, methods of staining and data analysis. Evaluation of the reproducibility of the technology with respect to its accuracy and error limits was necessary. In the past, several observations have been made by others that have not been substantiated by careful investigation. To accomplish our aims of determining CD4+ monocytes, it is necessary to look at quality control issues in flow cytometry to

validate the data.

The first step in the plan is to standardize the methodology for measurement of surface CD4 in monocytes from both normal persons and patients with HIV-1 infection so that we can give a definitive answer to the question of how many monocytes are CD4+, and if there are changes in fluorescence intensity due to disease progression in HIV-1 infected patients. Another question relates to the other target cell, the CD4+ T cell. Although their numbers decline with disease progression, it is not very clear how many of the circulating T cells are HIV-infected. It is known that HIV infection in vitro leads to down-modulation of the CD4 receptor. It is not known if CD4 is down-modulated in T cells from HIV-seropositive patients. The fluorescence intensity of CD4+ T cells will be measured in a cohort of HIV-infected patients to determine if all cells show decreased cell-surface CD4 or if only a subset of the cells do. Several investigators have shown that only a fraction (1% or less) of CD4+ T cells in blood are infected. Thus, if it can be shown that all T cells have decreased CD4, that means that mechanisms other than cellular infection are responsible for the decreased expression of CD4.

In addition, we are interested in the functional role of cell adhesion molecules (CAM) in monocytes/macrophages. In particular, we want to determine if maturation from monocyte to macrophage is associated with CAM surface expression changes and if such changes can be influenced or induced by the presence of exogenous hemopoietic growth factors.

1.5. OBJECTIVES

In summary, the objectives are:

- 1) Measurement of
 - i) the frequency of CD4+ monocytes from total leukocytes in normal donors and HIV-1 seropositive patients.
 - ii) surface CD4 expression on monocytes and T cells in normal donors and HIV-1 seropositive patients. The surface expression, represented by CD4 fluorescence intensity, on these two cell types will be correlated with disease progression.
 - iii) the surface expression of cell adhesion molecules (CD11/CD18) of the Integrin supergene family during maturation from monocytes to macrophages *in vitro* and to determine the effect of exogenous hemopoietic growth factor stimulation on their expression.

- 2) To identify
 - i) technical considerations relating to flow cytometric measurements to ensure reproducibility, accuracy and reliability of data.

II. MATERIAL AND METHODS

II.1. Source of Material (Blood Samples)

Human venous blood samples were obtained from:

- a) Volunteer laboratory workers (Federal Centre for AIDS, Health & Welfare and University of Ottawa Departments of Microbiology/Immunology and Physiology).
- b) Canadian Red Cross (units of citrated whole blood).
- c) Patients (HIV+) involved in Ottawa General Hospital (OGH) and Children's Hospital of Eastern Ontario (CHEO) HIV cohort study. Collaborating physicians were Drs. W. Cameron (OGH) and F. Diaz-Mitoma (CHEO).

II.2. Cell Separation

Preparation of Mononuclear Cells by density-gradient centrifugation on Ficoll-Hypaque:

- a) Whole blood (Red Cross Unit, 500ml) was separated into two 250ml flasks and centrifuged for 20 minutes at 1500 rpm to separate plasma and red blood cells.
- b) The interface was collected and 25ml aliquots were put into 50ml tubes, 12ml of PBS was added and the tube gently mixed and underlayered with 13ml of Ficoll. The tubes were spun for 30 minutes at 1800 rpm.
- c) The buffy coat was collected and washed 3 times with PBS, counted and either stained for flow cytometry, or resuspended in growth medium (10%FCS/IMDM) to 6×10^6 /ml.

II.3. Cell Staining and Analysis by Flow Cytometry of Peripheral Blood Leukocytes.

II.3.i Staining of Fresh Cells

Fresh cells were stained from either (1) unseparated whole blood after automated erythrocyte lysis using the Coulter Immunoprep system, or (2) after preliminary separation of mononuclear cells by density gradient (as described in II.2 above).

The Coulter Immunoprep Epics Leukocyte Preparation System was used to stain fresh whole blood. Staining, lysis and fixation are performed in a single tube inside the instrument, thus biohazard exposure is minimized. 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. The whole process with vortexing between reagent additions took 35 seconds plus the 15 minute incubation time. This method also eliminated the time-consuming and frequently difficult method of density gradient centrifugation to prepare mononuclear cells but did not exclude granulocytes; it decreased variability due to manual handling of sample.

Procedure:

- a) Human blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vacuum collection tubes by venipuncture.
- b) 100µl anti-coagulated whole blood was pipetted into 12 x 75 mm test tubes.
- c) Appropriate amounts of antibody(s) were added (determined from previous titration studies).
- d) Samples were incubated for 15 minutes at room temperature and placed into the Q-Prep instrument using the 35 s Cycle.

e) Samples were washed twice with PBS and resuspended in 2% paraformaldehyde in PBS and the samples were refrigerated until analysed on flow cytometer.

II.1.3.ii Staining of Cultured Cells

Cultured cells were stained in micro-tubes after first collecting and washing the cultured cells and pre-incubating them with saturating amounts of prepared heat-aggregated gamma globulin (procedure described in section II.5.ii). Monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were usually used (except for the cell adhesion molecules; see text). 10ul of the antibody was incubated with approximately 5.0×10^5 - 1.0×10^6 cells for 20-30 minutes at 4°C after which the cells were washed twice with PBS, resuspended in 2% paraformaldehyde in saline and kept at 4°C until analyzed on the flow cytometer. Higher fluorescence background staining due to nonspecific binding by Fc receptors made isotype controls mandatory.

II.3.iii Flow Cytometric Analysis of Stained Peripheral Blood

Daily instrument calibration and standardization was performed to obtain consistent results (e.g. AutoCOMP, QuickCal and QC3 beads)

Flow cytometric measurements were performed on a FACScan flow cytometer (Becton-Dickinson, Mt. View, Ca). This instrument is equipped with an air-cooled argon-ion laser operating at 15 mWatt at 488 nm (which is optimal for excitation of FITC and PE simultaneously) and with standard three color fluorescence detector photomultiplier tubes. The optical filters were factory-set standard configuration, with green fluorescence through a 530 ± 30 nm bandpass, orange fluorescence through 585 ± 42 nm bandpass, and red through a 650nm longpass filter to collect signals from FITC,

PE and PerCP (not used) respectively. Signals from light scatter channels were collected in linear mode, and signals from the three fluorescence detecting PMTs were collected in log. Acquisition and analysis were done with the FACScan Research Software (Becton-Dickinson) and a minimum of 10,000 cells were collected for each test and stored in list-mode using BDIS Research Software (version 2.1) on a Hewlett-Packard 9000 series model 310 PC (Hewlett-Packard, Fort-Collins, Co. U.S.A.).

Monocytes were distinguished from lymphocytes and granulocytes on the basis of their light-scattering ability (forward- and side-light-scatter) on a bivariate display. An electronic gate, set around the cell population bearing the light-scatter characteristics of monocytes (and/or lymphocytes), was activated for the analysis. The data were analyzed as logarithmic fluorescence histograms of scatter-gated cells. Mean fluorescence intensity was used as a measure of the amount of antibody binding cell surface receptor.

II.4. Instrument Calibration and Standardization

II.4.i Daily Alignment

The instrument was aligned daily to obtain the optimal resolution by using the AutoCOMP software (Becton-Dickinson). AutoCOMP is a menu-driven program that adjusts the photoelectric components (gain settings and fluorescence compensation) of the FACScan flow cytometer to known standards. Using AutoCOMP daily ensured that the flow cytometer recorded the intensity of events consistently. This allowed accurate comparison between data collected on different days.

AutoCOMP uses CaliBRITE beads (supplied by the manufacturer, Becton-Dickinson) as its reference standard, which are plastic microspheres unstained and labelled with FITC or PE.

AutoCOMP performs 3 FACScan adjustments: 1) gating singlet events, 2) adjusting photomultiplier tube (PMT) gain, and 3) fluorescence compensation.

1) Gating Singlet Events

The FACScan measures physical and biochemical properties of single cells. AutoCOMP sets upper and lower boundaries (or gates) for forward-scatter (FSC) intensity to minimize the effects of sample contamination. Once in place, these gates eliminate data from particles (debris or doublets) which do not fall within the set light-scatter limits.

To find the upper and lower thresholds for singlet events, AutoCOMP automatically acquires and analyzes 5000 events from unstained beads for the FSC parameter, using a preset lower threshold of 350. The program sets the upper and lower gates by statistical analysis of the resulting frequency histograms.

2) Adjusting Photomultiplier Tube (PMT) Gain

PMTs have multiple gain settings which influence their detection sensitivity. Increasing the gain results in greater amplification of a signal and alters its channel distribution. In automatic mode, AutoCOMP sets the PMT gain so that the unstained calibration beads distribute with a mean channel of 25 ± 2 for the fluorescence detector channels and 125 ± 2 for the SSC (side-scatter) detector channel. The FSC photodiode does not have multiple gain settings and is unaffected by this procedure.

3) Fluorescence Compensation

FITC and PE have overlapping emission spectra. While the emission maximum of FITC (520nm) and PE (575nm) are separated by 55nm, both fluorochromes emit light between 540 and 565nm. Despite the use of filters, some FITC radiation reaches the PE detector and some PE radiation reaches the FITC detector. AutoCOMP compensates for this remaining spillover by electronically subtracting the unwanted signal from each detector.

The standard sample for this compensation procedure is a mixture of unstained, FITC-stained, and PE-stained CaliBRITE beads. AutoCOMP determines the mean channel of the unstained beads for both PE and FITC parameters, and

electronically aligns these channels with the PE mean channel for the FITC beads and the mean FITC channel for the PE beads. This adjustment shifts unwanted fluorescence signals into the positions corresponding to autofluorescence signals.

II.4.ii Setting Target Conditions

After aligning the flow cytometer, a representative sample is run and the instrument settings adjusted to obtain light-scatter and fluorescence patterns which present the data as you wish for that type of sample preparation. The remainder of those samples should be run at these instrument settings.

II.4.iii Determining Target Channels

To determine target channels, the reference standards (using QC3 beads from FCSC) were analyzed at the same instrument settings as the target conditions for parameters of interest (forward- and side-scatter, FL1 and FL2). The peak (median) channels for each parameter of are recorded and these peak channels are now the target channels for this sample type.

QC3 standards are uniform microbeads which are surface-labelled with FITC and R-PE. When using FITC and R-PE labelled specimens, QC3 allows standardization of the data quickly and efficiently by setting target channels for light-scatter and fluorescence parameters in one instrument run. This serves to correct for variations in instrument performance (a source of error when analyzing samples) allowing comparison of results from samples run on different days and by different operators.

II.4.iv Re-establishing Target Conditions

In subsequent runs, the QC3 reference standards are set in the Target Channels to reproduce the Target Conditions.

Each time this sample type is to be analyzed on the flow cytometer, the QC3 reference standards are run and the instrument settings are adjusted (e.g., amplifier gains and PMT high voltage), so that the scatter and fluorescence peaks fall in their respective Target Channels.

II.4.v Determining Instrument Sensitivity

QuickCal™ (FCSC, Research Triangle Park, NC) is a quality assurance system for flow cytometers and is run daily after QC3 runs. It consists of a bottle of pre-mixed Quantitative Fluorescein Standard Microbeads and software which can be run in the computer of the flow cytometer.

The fluorescein microbeads have spectra that match cells labelled with antibodies conjugated to FITC. The microbead mixture contains six populations of microbeads which are all the same size, five populations labelled with FITC which differ in fluorescence intensity, and a blank unlabelled population. The range of these fluorescence intensities covers most samples found in the clinical setting. The software (1) generates a linear regression plot and statistical data to assess instrument linearity, (2) determines fluorescence threshold sensitivity, and (3) creates a quality assurance file of daily instrument performance. QuickCal is a powerful tool for monitoring and optimizing fluorescence intensity measurements.

*Detailed protocols of sample staining and of the daily instrument calibration (including AutoCOMP, QC3 and QuickCal procedures) are found in Appendices I and II respectively.

II.5 Cell Culture for Monocyte/Macrophage CD11/CD18 Cell Adhesion Molecule Study

II.5.i Culture

A mononuclear cell suspension at a concentration of 5ml of 6×10^6 /ml was added to 25 cm² tissue culture flasks. After 45 minutes incubation at 37°C, the nonadherent cells were removed, the flask washed and then growth medium supplemented with growth factors [GM-CSF, M-CSF and IL-3 (from Genzyme) at 50Units/ml] was added. At intervals, the cultures were terminated by detaching adherent cells with PBS/EDTA treatment and washing.

II.5.ii Staining and Analysis for CD11/CD18 Markers

Heat-aggregated Human Gamma-Globulin (AHGG) is used to reduce background by nonspecific binding of antibodies to Fc receptors present on the monocyte and granulocyte membrane.

Cells at approximately 1×10^6 /Eppendorf tube were washed once in PBS, incubated in 100-200µl AHGG for 30 minutes at 4°C. The cells were then washed once in PBS and stained as usual (see II.3.ii).

Preincubation with AHGG was done each time cultured cells were stained for flow cytometric analysis. When staining fresh cells, it was not necessary. AHGG was prepared by heating crude monomeric IgG to induce self-association and was prepared as follows:

a) 200µl of a 50mg/ml Human IgG stock (Sigma) was added to 300µl PBS and kept at 60°C for 15-20 minutes.

b) 4.5ml 0.02% Sodium Azide/2% BSA/PBS was added.

c) A saturating amount of this mixture was added to each sample and left for 30 minutes at 4°C, after which the samples were washed once with PBS before staining. The prepared AHGG should ideally be used fresh, stored at 4°C and discarded after one week.

After incubating with saturating amounts of heat-aggregated IgG (to block Fc receptors) for 30 minutes at 4°C, the cells were washed and stained by indirect immunofluorescence; 10µl of unconjugated monoclonal antibody (CD11a/CD18, CD11b/CD18, CD11c/CD18 and CD18, from DAKOPATTS) were added to approximately 1×10^6 cells and incubated for 25 minutes at 4°C. 50µl of biotinylated sheep-anti-mouse F(ab) secondary antibody (1:40 dilution) was incubated with the cells for 30 minutes at 4°C and washed twice with PBS. The cells were incubated with Streptavidin-phycoerythrin (Jackson Laboratories) at a 1:200 dilution, for 20 minutes at 4°C, washed twice, fixed in 2% paraformaldehyde, and stored at 4°C until analysis with the flow cytometer (FACScan).

II.6. Statistics

Paired Student t-tests were used to determine whether the means were significantly different at the $p = 0.05$ level. To determine correlation between CD4 surface expression and % of CD4+ T cells, a Pearson correlation coefficient was calculated and the significance was tested by finding the t value.

Various statistics were computed by the FACScan system (FACScan Research Software). Quadrant statistics were computed by splitting the dot plot into four rectangular regions which separate the populations. Another type of statistic was

computed from single parameter (histogram) data. For the calculation of the statistics for log data, the channel number is converted to its corresponding linear value before any calculations are performed. After the values have been computed, they are converted back to log channel numbers. "Percent" refers to the percent of gated events within the region. "Mean" refers to the average channel value of the region defined by markers.

III. RESULTS

There is no agreement about the frequency and antigen density of CD4 in or on the surface of blood monocytes, and the effect of HIV-1 on CD4 density is also not clear. Before attempting to answer these questions directly, various preliminary experiments were performed pertaining to the antibodies and staining methods. To ensure accuracy, reproducibility and reliability of data, a new calibration system was implemented to monitor instrument (i.e. flow cytometer) performance.

III.1. Preliminary Experiments Ensuring Reliability of Data

III.1.i CD4-PE vs CD4-FITC

Because CD4 is present at low antigenic density on monocytes (Stewart et al, 1990), its accurate detection using fluorochrome-conjugated antibodies has been difficult. In addition, monocytes have higher autofluorescence and nonspecific fluorescence when fluoresceinated irrelevant antibodies are used as controls (non-specific binding to FcR). Under such conditions, the population of positive cells overlaps the control population and the frequency of the positive cells tends to be severely underestimated. The correct choice of fluorochrome is important for detection and to obtain the best separation between positive and negative cells. The first such experiment involved determining the best fluorochrome: fluorescein-isothiocyanate- (FITC) or phycoerythrin(PE)-conjugated antibodies.

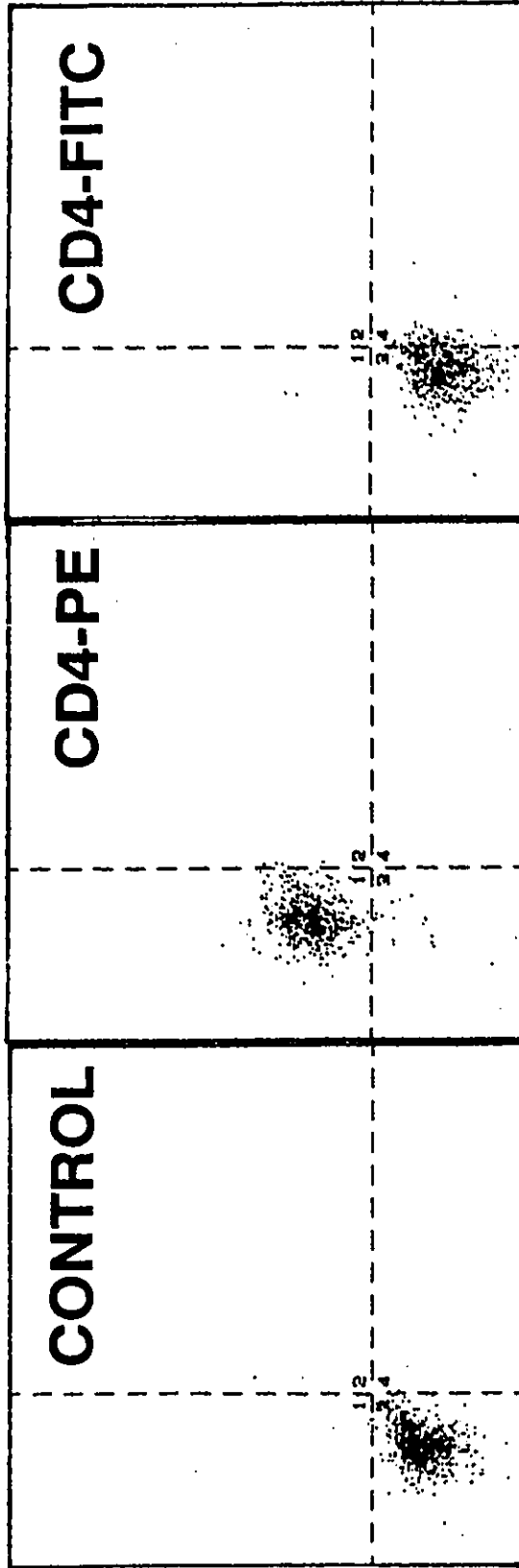
The experiment consisted of phenotyping fresh blood with either CD4-PE/CD14-FITC (n=42), CD4-FITC/CD14-PE (n=8), or CD4-PE and CD4-FITC (n=3) separately. The samples were processed using the Q-Prep unit; CD14 is a monocyte-specific marker.

Without exception, the PE-conjugated anti-CD4 gave a clear separation between positive and negative cells. Figure 7 represents samples stained with isotype controls and

either CD4-PE or CD4-FITC and analyzed using Quadrant statistics. The CD4-FITC gave a weak separation that overlapped with the cells' autofluorescence, and in fact the majority of cells were negative. Using CD4-FITC, $32\% \pm 15$ (n=8) were CD4 positive, compared to PE with $97\% \pm 2$ (n=42).

The fact that FITC was the tag frequently used in earlier research may account for the variability previously reported in the literature.

Figure 7: CD4-PE vs CD4-FITC. Determination of the best fluorochrome [fluorescein isothiocyanate (FITC) or phycoerythrin (PE)]-conjugated monoclonal anti-CD4 antibody. The PE-conjugated monoclonal antibody clearly gave the best separation between positive and negative cells (Quadrant 3), while CD4-FITC overlapped with negative cells. X-axis measures green fluorescence (FL1, FITC) and the Y-axis measures red fluorescence (FL2, PE).



PE (FL2)

FITC (FL1)

III.1.ii Effect of Antibody Concentration

Standardization of each reagent must be performed to determine both specificity and sensitivity of the system. The critical test used to determine quantity of antibody is termed the plateau test and involves serial dilution of the staining reagent and assessment of the percentage and type of cells stained. Analysis involves plotting percentage of stained cells versus increasing concentration of antibody used. When saturation is achieved, a plateau is reached where the percentage and intensity of stained cells is constant and independent of antibody concentration. All immunofluorescence should be performed within this working range.

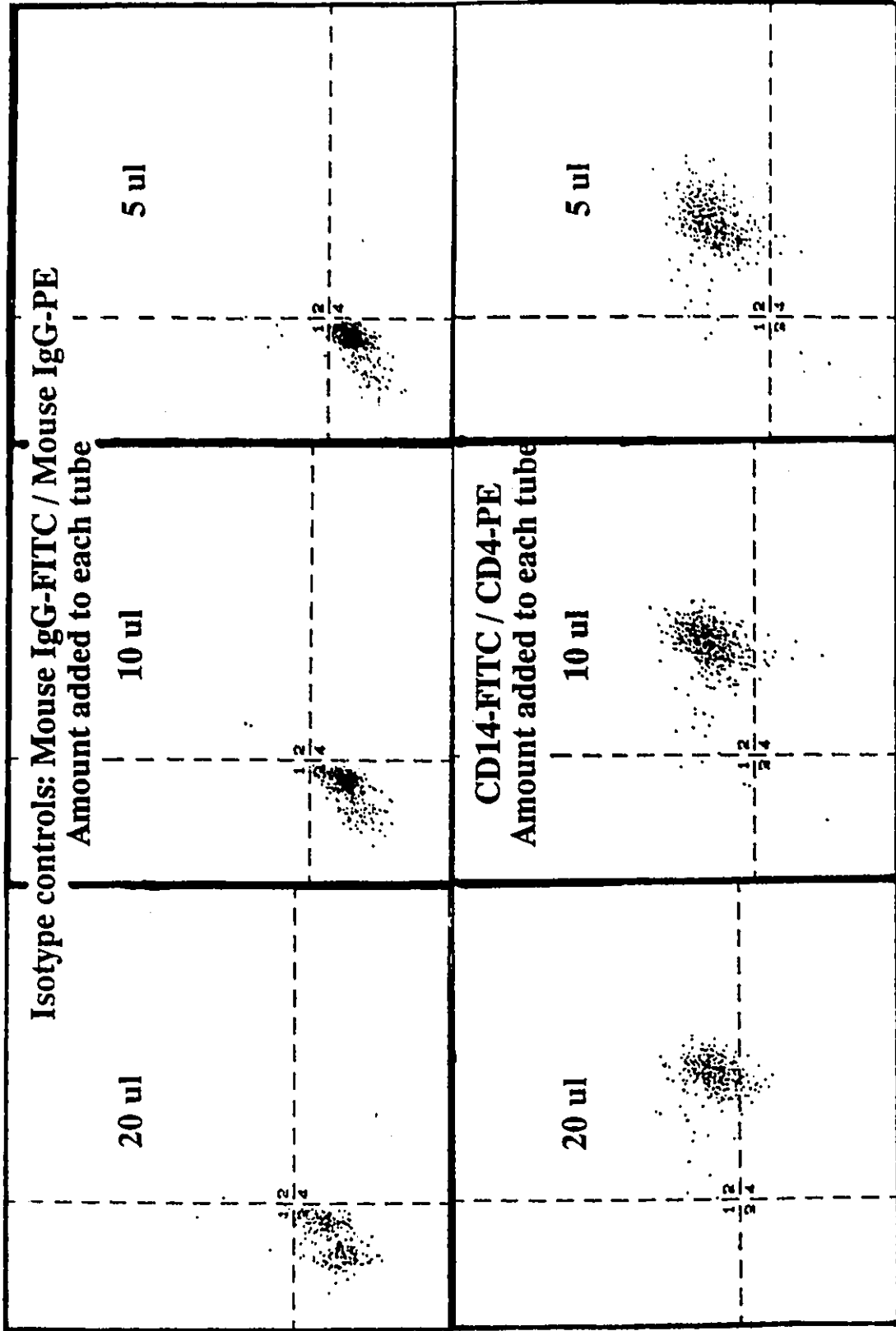
As the concentration of antibody is increased, further "nonspecific" effects may become evident in which additional cells are stained because of a variety of physicochemical or immunologic phenomena. The latter include Fc receptor interactions through formation of immune complexes that secondarily bind to cells expressing Fc gamma receptors. Supersaturation with antibody may not reflect true binding, and when staining with isotype controls the cursors may be set with fluorescence not representative of true random binding. There is also a donor effect, as different people, especially patients, have different affinities for the isotype controls.

To determine true random binding, the isotype controls and other monoclonals must be titrated out by diluting the monoclonals. Briefly, fresh whole blood was incubated with decreasing amounts of monoclonal antibody: (20 μ l, 10 μ l and 5 μ l) and processed in the Q-Prep Processing Unit. Results were analyzed on the FACScan Flow Cytometer.

Fig. 8 clearly shows that with decreasing amount of antibody, from 20 μ l to 5 μ l, the isotype control became a tighter population and the cursor settings decreased. This affected subsequent analysis as the isotype control stained samples were the first to be analysed and the cursors were set from the background autofluorescence. The cursor

separates positive from negative cells. So as not to compromise positivity, 10 μ l of antibody was chosen to use routinely when staining monocytes, as opposed to the standard 20 μ l currently used for lymphocytes as suggested by the manufacturer.

Figure 8: Antibody Titration. Titration of antibodies on monocyte-gated human peripheral leucocytes. The figure demonstrates the importance of isotype control dilutions as these set the cursors for subsequent analysis. Oversaturation increases nonspecific binding and does not reflect random binding. This artificially raises the cursor settings, thereby compromising positivity in further analysis.



PE (FL2)

FITC (FL1)

III.1.iii Effect of Time and Light Exposure on Fluorescence Intensity and Positivity

After studying flow-cytometric data it becomes apparent that results are frequently not reproducible, and that they vary from day to day and from test group to test group. Therefore experiments were set up to analyze this variability and to determine why samples stained in exactly the same way and by the same person give close but not identical values.

The person staining the samples may not always be the same person running the samples on the flow cytometer, and it was realized that frequently there is a lag time of hours to days before the samples are analyzed after staining. This is compounded with frequent long light-exposure time as samples are often left on the bench while waiting to be analyzed. It was therefore decided to monitor the stability of cell-bound monoclonal antibodies after staining and before running samples.

The monoclonal antibody (mAb) used was anti-CD4 conjugated to PE as this is the mAb most often used and of most interest for the present work. The anti-CD4 antibodies from two companies [Becton-Dickinson (Leu3a) and Coulter Electronics (T4RDI)] were compared for the effect of time and light-exposure on fluorescence intensity and positivity on T cells.

Figure 9A shows results from both companies' anti-CD4, with Coulter's anti-CD4 exposed to light (bench) and covered (dark) at room temperature against time (0 to 240 minutes). Fluorescence intensity is represented by the number of channels between positive and negative peaks. At time 0, Becton-Dickinson's Leu3a-PE has a greater channel separation compared to Coulter's. This means Leu3a-PE gives a better separation between positive and negative cells which is particularly important since CD4 is expressed at low surface density and the signal frequently overlaps with negative cells.

It is also obvious from this graph that the signal from both antibodies fades with time and with exposure to light. There is some loss of signal from Coulter's T4RD1 even

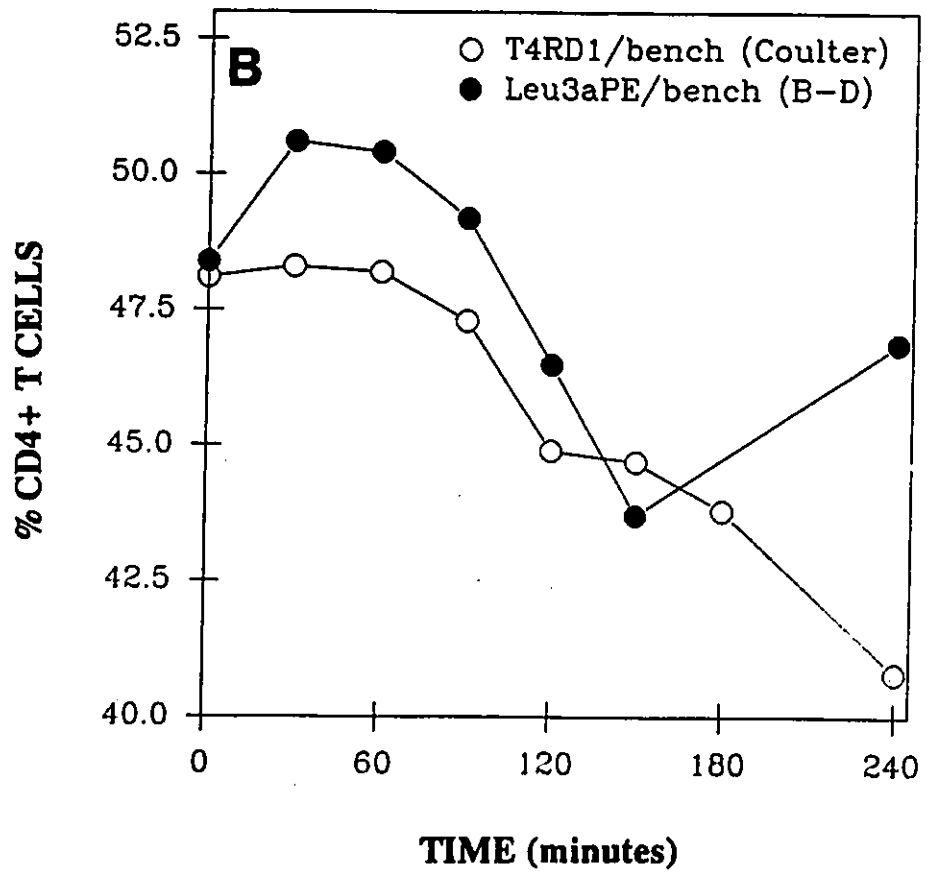
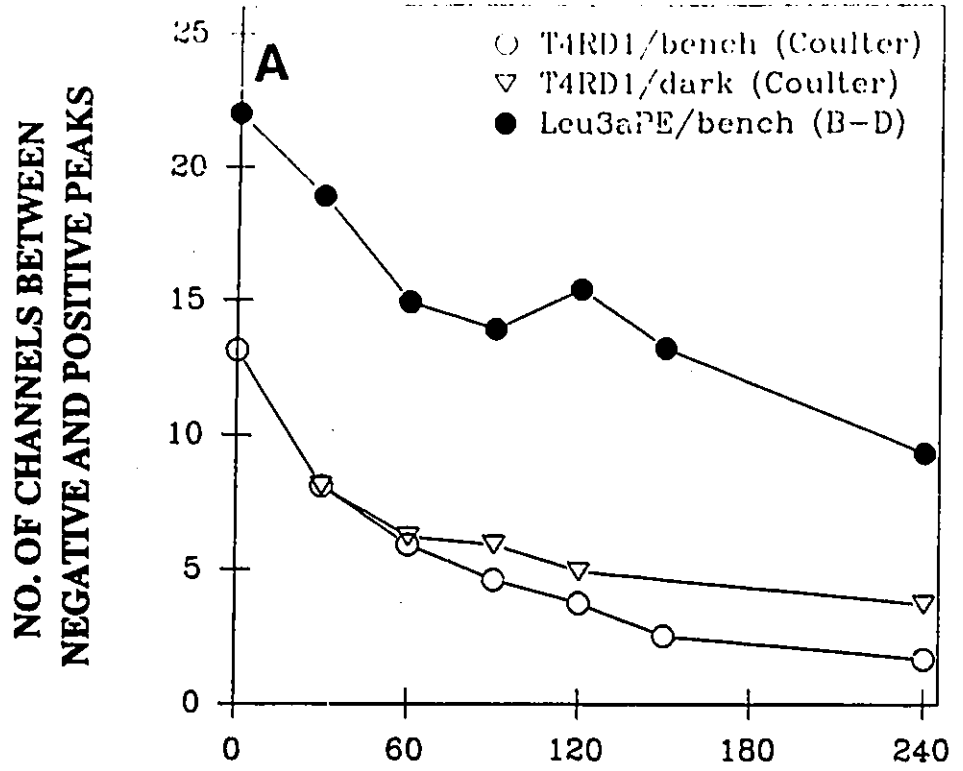
if kept in the dark, but the drop is not as severe as the sample exposed to the light on the bench.

With time and exposure to light, another factor being compromised is positivity (fig. 9B). It appears that 2 hours maximum can be tolerated before the signal is affected beyond the $\pm 2.5\%$ range of acceptance.

These experiments serve to emphasize the fact that every variable must have a corresponding control in flow cytometry. This includes :

1. Daily standardization and calibration of the instrument
2. The company the antibody was purchased from
3. The conjugated tag (FITC or PE)
4. The amount of antibody used to stain
5. The waiting time between staining and running of the sample
6. Light exposure

Figure 9: Stability of Cell-Bound Phycoerythrin-Labelled Monoclonal Antibodies:
A: The number of channels between negative and positive peaks was graphed against time (0-240 minutes). Two anti-CD4 monoclonal antibodies were tested [Leu3a from B-D (Becton Dickinson) and T4RD1 from Coulter]. **B:** The percentage of CD4+ T cells was graphed against time (0-240 minutes). The effect of time and light-exposure on fluorescence intensity (A) and positivity (B) were investigated and both have a negative effect. * Experiment was performed by Michele Bergeron at Health & Welfare, Federal Centre for AIDS.



III.I.iv. Instrument Calibration

Immunophenotyping by flow cytometry can provide fluorescence intensity information reflecting the amounts of labelled antigen on the surface of the cells analyzed. Appropriate quality control procedures must be used to control for instrument and biological variables such as instrument performance and sample preparation and storage which influence the sample's fluorescence signal.

The goal of quality control in any process is to ensure accurate and reproducible results. In flow cytometry, accuracy is a difficult question because there are at present no absolute standards against which to validate commonly measured cellular properties. Reproducibility is a function of both biological factors (specimen type, preparation process, reagent stability, etc.) and instrumental factors (optical alignment, filter, signal:noise ratio, etc). By adhering to strict procedural protocols and not deviating from them, the first function of reproducibility can almost be guaranteed.

Data must be comparable and to achieve this, particle standards (QC3 beads from Flow Cytometry Standards Corporation) were implemented in the standardization. The purpose of particle standards in flow cytometry is to ensure that data taken at different times and possibly on different instruments can be compared quantitatively.

A major source of error which can influence comparison of flow cytometry data is difference in instrument performance. Proper reference standards can be used to correct for differences in fluorescence, as well as light-scatter signals. Such reference standards have the same size and fluorescent dyes which yield the same excitation and emission spectra as the samples. The microbead reference standards were developed to facilitate instrument performance and are very accurate.

An instrument calibration process was developed to optimize and monitor flow cytometer adjustments and to assess precision, accuracy and sensitivity and to ensure reproducibility of the instrument. This fluorescence standardization involved four major

steps: 1) Alignment 2) Setting Target Conditions 3) Determination of Target Channels and 4) Re-Establishing Target Conditions.

1. Alignment: The instrument (FACScan Flow Cytometer, Becton-Dickinson) was adjusted to obtain optimal resolution. This was achieved by daily calibration using CaliBRITE Beads (Becton-Dickinson) and a menu-driven software program (AutoCOMP) that adjusts photoelectric components of the FACScan to known reference standards; CaliBRITE beads are plastic microspheres unstained and labelled with FITC or PE.

2. Setting Target Conditions : After aligning the flow cytometer, a representative sample was run and the instrument settings (amplifier gains and PMT high voltage) adjusted to obtain light-scatter and fluorescence patterns which present the data as desired for that type of sample preparation. The representative sample was stained for lymphocyte subpopulations, CD4 and CD8.

(Steps 3 and 4 were implemented later on in the study when a more thorough calibration and standardization was needed. This only became apparent after preliminary experiments were done, and is expressed in the Results as pre- and post-QC3 implementation, Table 7)

3. Determination of Target Channels: After optically aligning the flow cytometer, the remainder of the specimens should be run at these instrument settings.

The QC3 reference standard microbeads from FCSC (Flow Cytometry Standards Corporation, Research Triangle Park, North Carolina) are uniform microbeads which are surface-labelled with FITC and R-PE. The QC3 microbeads were run on the FACScan after the target conditions instrument settings were determined, and the peak (mean) channels of each parameter of interest were recorded; these became the target channels

for this sample type. The peak (median) channel was recorded for each parameter of interest (FL1 and FL2).

Each time a sample of this type was analyzed on the flow cytometer, the QC3 reference standards were run and the instrument settings adjusted, i.e. amplifier gains and PMT high voltage, so that the scatter and fluorescence peaks fell in their respective Target Channels.

The target conditions (user-specific conditions for analyzing samples) were the same for all samples. The target channels (the peak histogram channel for a given parameter which is obtained when analyzing a reference standard under appropriate target conditions) were 691 for the FL1 channel (median) and 767 for the FL2 channel (median). The instrument was adjusted each time samples were run so the scatter and fluorescence peaks fell in these target channels.

4. Re-establishing Target Conditions: In subsequent runs, the QC3 reference standards were set in the target channels to reproduce the target conditions. Each time this sample type was to be analyzed on the flow cytometer, the QC3 reference standards were run and the instrument settings adjusted so that the scatter and fluorescence peaks fell in their respective target channels.

Quickcal (FCSC, Research Triangle Park, NC) was run immediately following the QC3. This also uses labelled beads and is supplied with a software program to determine the instrument sensitivity.

This whole procedure corrected for variations in instrument performance, thus allowing comparison of results from samples run on different days and by different operators.

The staining protocol was the same as described below for CD4+ phenotyping except that the flow cytometer was calibrated daily to obtain a relative measure of antigen density.

Note: see detailed daily protocol for instrument calibration in Appendix II

III.2. CD4 Measurement

III.2.i Percentage of CD4+ Monocytes in Normal Donors and HIV+ Patients

To resolve the controversy as to whether monocytes are CD4+, and to compare normal values to HIV+ samples, we used a whole-blood, two-color or single-color immunofluorescence method and flow cytometry for cell analysis. Blood cells were stained with antibody mixtures (CD3, CD4, and CD8 for T cells and subsets; CD14 and CD4 for monocytes) labelled with FITC or PE; CD3-FITC:CD4-PE, CD3-FITC:CD8-PE, CD14-FITC:CD4-PE, CD4-PE alone, and labelled isotype controls. The operational definition (for this study) of a monocyte was a cell identified by its location on the light-scatter diagram, and that was CD14+.

Table 6 shows results from light-scatter gated fresh monocytes and T cells stained using CD4-PE with CD14-FITC or CD4-PE alone using the Quick Immunoprep System (Coulter Electronics). The monocyte results are expressed as the percentage of double-labelled (CD14+/CD4+) cells as a percentage of total CD14+ cells. From 42 normal samples, 97% \pm 2.3 were CD4+ monocytes. From the original 48 samples, 6 were beyond two standard deviations from the mean and were excluded; they ranged from 85-89%.

Of the 42 normal samples, 28 were singly-stained with CD4-PE only (the outlying values from the first column were not part of this group). 97% \pm 1.6 were CD4+ monocytes. This result is not significantly different ($p > 0.05$) from the double-stained (CD14/CD4) result, and suggests that CD4 can effectively replace CD14 as a monocyte marker, if the CD4 antibody is conjugated to PE.

To determine the effect of HIV infection on CD4 positivity of monocytes and to see if there was any correlation between disease and percent of CD4+/CD14+ monocytes, 45 double-stained (CD14/CD4) monocyte-gated HIV+ samples were phenotyped; 98 ±2.2% were CD4+ (there was no statistically significant difference between normal and HIV+ patients). Unlike monocytes, lymphocytes are affected by infection and the percentage of CD4+ T cells is used as a marker of disease progression. Of 60 normal lymphocyte-gated cells, 61 ±10% were CD3+/CD4+ (i.e. CD4+ T helper lymphocytes). Of the 82 HIV+ samples phenotyped, 25 ±17% of cells were CD3+CD4+. The HIV+ patients were in all stages of disease with lymphocytes ranging from 2 - 64% CD3+/CD4+ and this is reflected in the large standard deviation (±17).

From this, three major conclusions can be drawn. (1) Virtually all monocytes are CD4+. (2) There is no statistical difference between the two methods of analysis of CD4 on monocytes. The recommendation from this experiment is that CD4 can effectively and reliably replace CD14 as a monocyte marker which eliminates double-staining for CD14 and CD4 and (3) There is no effect on the concentration of CD4+/CD14+ monocytes by HIV infection, unlike CD4+ T cells whose numbers decline with disease progression.

TABLE 6
CD4 ON HUMAN MONOCYTES AND T CELLS

Cell Selection Based on Light-Scatter

	NORMALS		HIV+ PATIENTS	
	n	%	n	%
MONOCYTES:				
Two Color CD14+CD4+ Cells (% of total CD14+)	42	97 ± 2.3*	45	98 ± 2.2*
Single Color CD4+ Cells	28	97 ± 1.6*	N.D.	
T CELLS:				
CD3+CD4+ Cells (% of total CD3+)	60	61 ± 10	82	25 ± 17

Two-color immunofluorescence (CD14/CD4, or CD3/CD4) or single-color (CD4 alone) immunofluorescence was done and samples were run on the FACScan flow cytometer (Becton-Dickinson). There is no effect on the percentage of CD4+ monocytes by HIV infection between normals and HIV+ patients, compared to the marked decrease in CD4+ T cells in normals and HIV+ patients. Single-staining with CD4 demonstrated that CD4 could effectively replace CD14 as a monocyte marker (see monocyte two color normals and single color normals). (* no significant ($p > 0.05$) difference among values, N.D. not done)

III.2.ii Analysis of CD4 Fluorescence Intensity in T Lymphocytes and Monocytes: Effect of Target Channelling

Included in our second objective of standardizing the measurement of CD4 on monocytes, was to determine the Mean Fluorescence Channel Number (MCN), which is the average channel value of the region defined by the cursors or the Median Fluorescence Channel Number (MFC) which is the middle channel of the region. This value will give an idea of the antigenic density of CD4 on the surface of monocytes. HIV-infected blood samples will be run in parallel to compare CD4 density and the percentage of CD4+ monocytes.

To standardize the measurement of fluorescence intensity of CD4 on T lymphocytes and monocytes in normals and HIV+ patients, two-color microbeads (QC3) were used for the daily instrument calibration and compared with measurements done in normals and patients before and after QC3 was introduced in our lab.

QC3 is used in target channelling. Target channel (mean fluorescence) is first set up using a stained normal biological sample and QC3 beads as explained in the previous section. The mean fluorescence channel number (MCN) for both QC3 colors is recorded after manual fine-tuning of the instrument using the biological sample. The QC3 MCN becomes the target channel. In subsequent days, the instrument is adjusted using QC3 so that the same MCN (± 2 channels) is used every day.

Shown in Table 7 are MCN values of CD4-PE fluorescence from normal donors and HIV+ patients from monocytes and T cells before (pre) and after (post) the implementation of the QC3 beads in the daily instrument calibration protocol. The "distance" refers to the number of fluorescent channels between the T cell CD4 MCN and the monocyte CD4 MCN (T cell MCN - Monocyte MCN).

Comparing values from normal donors pre- and post-QC3, it is clear that values of the T cell MCN and monocyte MCN are significantly different. However, the standard

deviation was reduced by at least 65% post-QC3 implementation . The means of the distance values were not significantly different.

Within the HIV+ patient values, only the monocyte MCN values were not significantly different pre- and post-QC3. The standard deviations for the T cell and monocyte CD4 MCN were also not significantly different. The standard deviations after QC3 implementation are not significantly lower than in normals. This is not due to QC3 poor performance, but rather to the varying stages of disease in these individuals. We have shown that the distribution of CD4 MCNs varies with the percentage of CD4+ lymphocytes, and the large standard deviation is a reflection of this.

The most significant finding is that the distance between normal and HIV+ patient T cell and monocyte CD4 peaks shortens [209 \pm 28 to 157 \pm 40 (pre-QC3) and 201 \pm 10 to 95 \pm 24 (post-QC3)]. This is due to a decrease in fluorescence intensity in T cells with disease and an increase in CD4 fluorescence intensity on monocytes. These results are shown graphically in Figure 10.

TABLE 7

**TARGET CHANNELLING FOR THE ANALYSIS OF FLUORESCENCE
INTENSITY**

Use of 2-Color Microbeads (QC3)

MCN of CD4-PE Fluorescence					
	QC3	n	T cells	Monocytes	Distance
Normals	pre	30	696 ± 30	487 ± 40	209 ± 28*
	post	10	638 ± 6	437 ± 11	201 ± 10*
HIV+	pre	47	671 ± 34	514 ± 41*	157 ± 40
	post	29	601 ± 34	507 ± 35*	95 ± 24

The CD4 MCN (surface antigenic density) was measured in normal donors and HIV+ patients on monocytes and T cells before (pre) and after (post) the implementation of QC3 beads. The distance (number of channels) between the T cell and monocyte CD4 peaks reduces significantly ($p < 0.05$) with HIV infection and is due to the decrease in fluorescence intensity of T cells and the increase in monocyte CD4 expression. (* no significant difference at 0.05 level, MCN Mean Fluorescence Channel Number)

III.2.iii Mean Fluorescence Channel Number (MCN) and Percentage of CD4+ T Cells on Normal Donors and HIV+ Patients

It has been reported that infection of T cells with HIV-1 leads to a reduction in the cell surface expression of CD4. We were curious to find out if there was any correlation between disease progression (measured by the percentage of CD4+ T cells) and the surface CD4 antigenic density. We also wanted to determine if there was a similar reduction in CD4 expression on monocytes.

Using the same immunophenotyping method (CD3-FITC:CD4-PE, CD3-FITC:CD8-PE) previously described, the CD4 MCN was recorded from T cells and monocytes. The percentage of CD3+/CD4+ (i.e. T helper lymphocytes) expressed as a percentage of total CD3+ cells was determined for each normal individual and patient using the Quadstat and the CD4/CD3 staining combination.

Fluorescence is always presented on a logarithmic scale. The computer converts log data to corresponding linear values, performs the calculation and then converts it back to log channel values that are arbitrary "channel" units (ranging from 0 - 1023) and these calculations are presented on a linear scale even though the values are logarithmic. In order to plot these logarithmic fluorescence MCNs on a linear scale, the logarithmic data was converted to linear values and these were plotted.

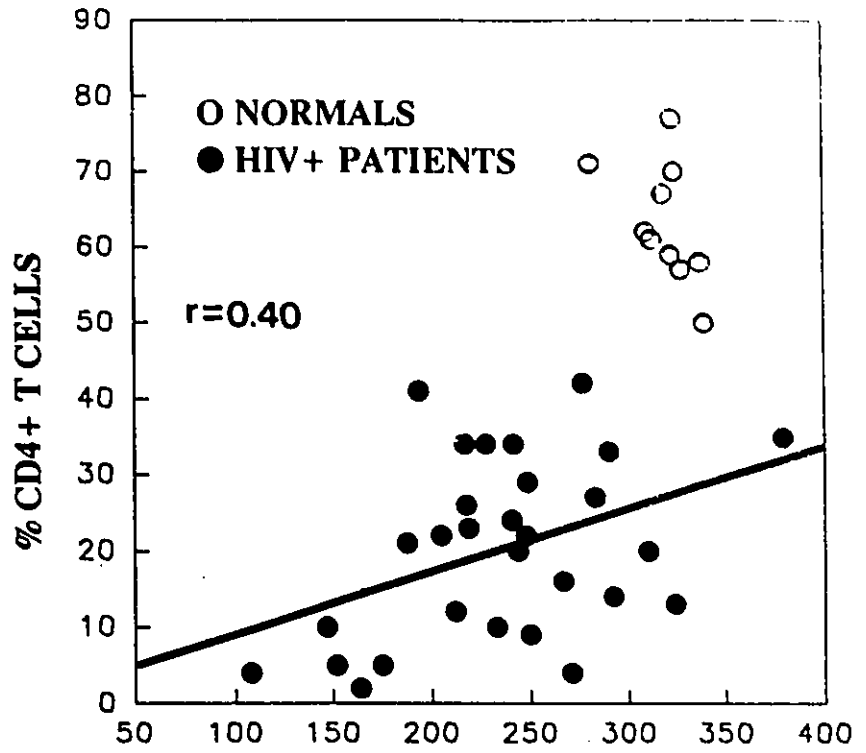
From Figure 10A, there is a clear difference between the CD4 MCN (x-axis) of CD4 in normals and in patients. Most HIV+ samples had a lower MCN than normals (linear values are 319 ± 16 and 235 ± 58 respectively), and greater variability in their CD4 expression. The CD4 distribution was widespread with some values in the monocyte range of fluorescence. In addition to this, the correlation between decreasing percentage of CD4+ lymphocytes with decreasing CD4 surface expression was significant [$r=0.402$ (Pearson correlation coefficient), $p<0.03$]. It is intriguing that the whole population of CD4+ T cells have this down-modulation of CD4 expression when clearly not all these

cells carry HIV proviral DNA (i.e. they are not all infected). The normals do not show this trend and stay clustered around 60-70% CD4+/CD3+ with a fairly consistent MCN.

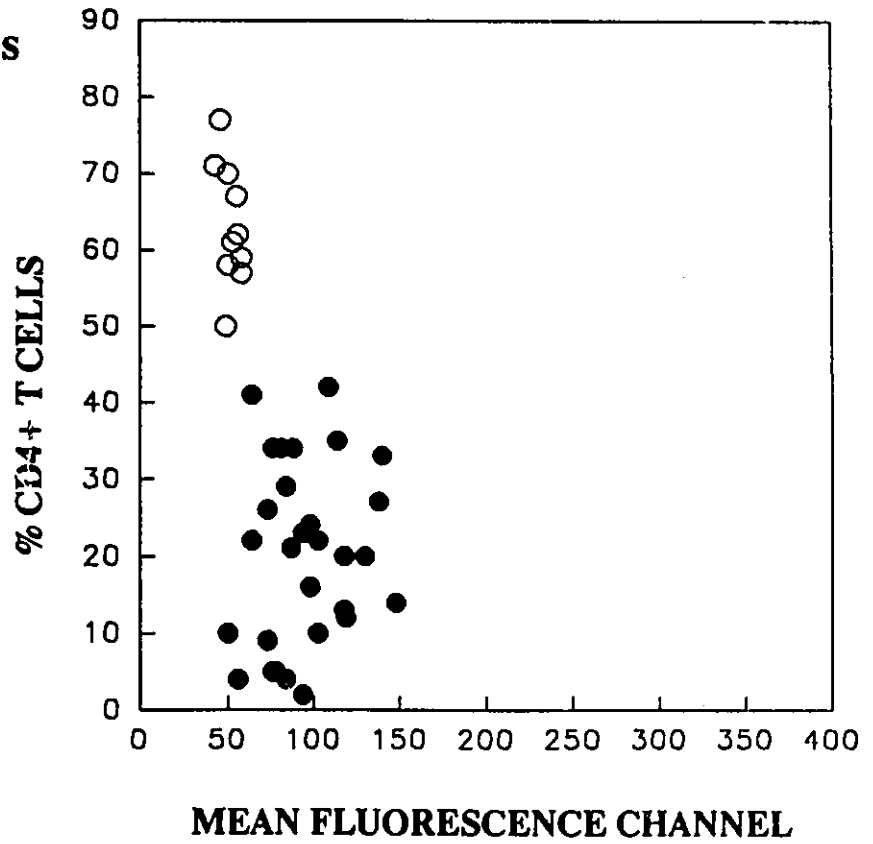
Figure 10B represents the same normal donor and patient samples as in 10A, except the MCN of monocytes was recorded and plotted against the percentage of CD4+ T cells to see if there was any modulation of CD4 on monocytes (as there appeared to be on T cells) with HIV infection. As expected, the normals stayed within a very narrow and tight range of channel vales (linear value 52 ± 5) with little variability. In patients, the variability in CD4 antigenic density is greater and increased in patients (linear value 95 ± 25). Since all CD14+ monocytes are CD4+, the y-axis was kept the same i.e. percentage of CD4+ T cells. There was no correlation of surface expression and disease stage in monocytes as demonstrated in T cells ($r=0.152$).

Figure 10: T Cell and Monocyte CD4 Fluorescence Intensity and Percentage of CD4+ T Cells. Graphs A and B demonstrate the relationship between CD4 fluorescence intensity (X-axis) on T cells (A) and monocytes (B) and percentage of CD4+ T cells, a measure of disease progression (Y-axis) on normal donors (n=10) and HIV+ patients (n=29). **A:** There is a distinct separation between the fluorescence intensity of T cell CD4 in normal and in HIV+ patients (linear values are 319 ± 16 and 235 ± 58 respectively, $p < 0.05$). Most HIV+ samples had a lower MCN than the normal donors and in addition to this, there is a correlation between decreasing percentage of CD4+ lymphocytes and decreasing CD4 surface expression ($r = 0.402$, $p < 0.03$). **B:** In HIV+ patients, the antigenic surface density for monocyte CD4 is increased in most patients when compared to normal donors (linear values are 95 ± 25 and 52 ± 5 respectively, $p < 0.05$)

A. T Cells



B. Monocytes



III.2.iv Light-Scatter and CD4 Fluorescence Patterns in HIV+ Patients

When attempting to gate on a specific cell population on the light-scatter diagram, it was observed in patient samples that frequently the cell populations overlapped. Often it was obvious that the sample being analyzed was a patient sample simply by looking at the light-scatter diagram.

Previously, by gating on both populations (monocytes and lymphocytes) and using the single histogram profile, both T cell and monocyte CD4 could be clearly distinguished. From this profile the percentages and mean fluorescence channel could be noted. This was true for most normals (97% of the time). When analyzing patient samples the single histogram peaks of CD4 for T cells and monocytes would frequently overlap which complicated cursor settings (see Figure 11 and Appendix III, Figure 15B).

Cytofluorometric analysis showed that 77% of HIV positive patients have abnormal light-scatter display. Lymphocyte gating under these conditions is very difficult. In addition, many of the patients presented an unusual CD4 fluorescence profile with CD4-PE. Due to the decreased antigen density on CD4+ T cells and increased CD4 antigen density on monocytes, there is an overlap with the CD4 fluorescence peaks.

We studied list-mode data from 56 patients using two-color immunofluorescence with CD3-FITC/CD4-PE and keeping the same gates (lymphocyte and monocyte), but using the Quadstat for analysis. Quadrant 1 contains the CD4+ monocytes (monocytes would be negative for CD3, a pan T cell marker). Quadrant 2 has the double-labelled CD4+ T cells (CD3+) and Quadrant 4 has CD3+CD4- T cells (probably CD8+ T cytotoxic cells). Quadrant 3 contains the negative cells (e.g. B cells and NK cells).

A variety of patterns were noted and illustrated in figure 11 and their frequencies are noted in Table 8.

In conclusion, an improved method was developed to analyse CD4 on T cells and monocytes in samples displaying abnormal light-scatter diagrams, normally occurring in

patient samples. This was achieved by including both lymphocyte and monocyte populations in the operator-defined gate and by using the CD3/CD4 staining combination. By using Quadstat for data analysis, the CD4+ monocytes (quadrant 1) were clearly separated from the CD4+ T cells (quadrant 2) without any population overlap.

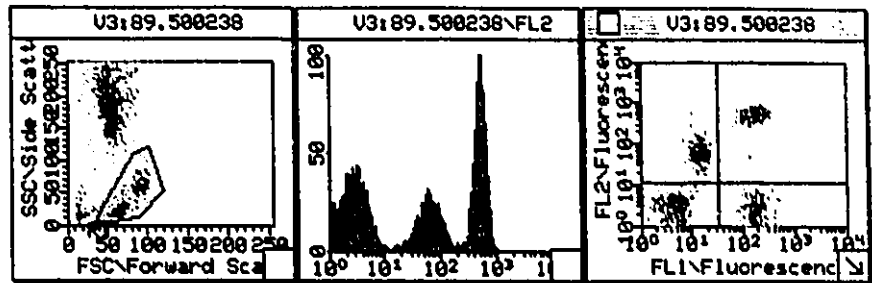
Figure 11: Light-Scatter and CD4 Fluorescence Patterns From HIV+ Patients. Frequently with patient samples, the various cell populations on the light-scatter diagrams overlapped, making gating and single population analysis difficult. By gating on both monocytes and lymphocytes (both CD4+) and using the CD3FITC/CD4PE staining combination and the Quadstat graph for analysis, clear distinctions could be made between CD4+ monocytes (Quadrant 1) and CD4+ lymphocytes (Quadrant 2). A=Normal light-scatter, Normal CD4 profile; B=Normal light-scatter, Abnormal CD4 Profile; C=Abnormal light-scatter, Normal CD4 profile; D=Abnormal light-scatter, Abnormal CD4 profile; 1=Light-scatter diagram; 2=CD4 fluorescence intensity profile; 3=CD3FITC/CD4PE Quadstat Analysis diagram.

1

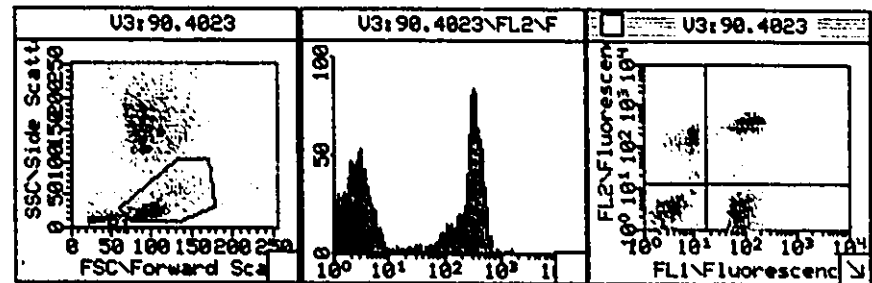
2

3

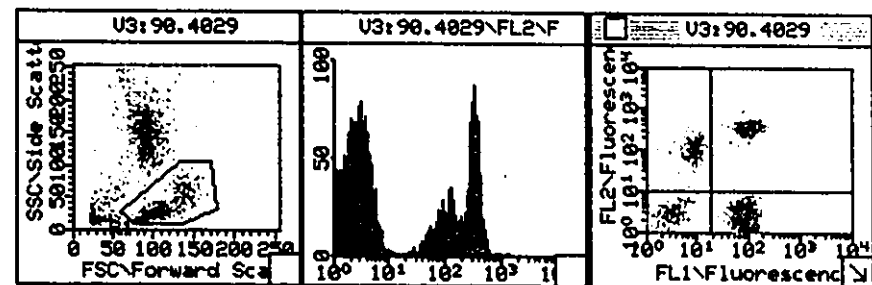
A



B



C



D

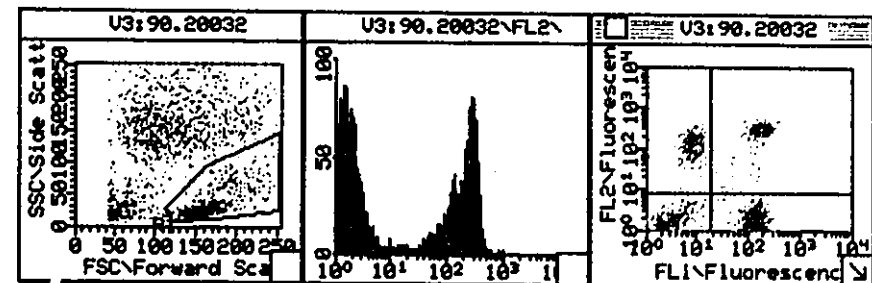


TABLE 8

LIGHT-SCATTER AND CD4 FLUORESCENCE PATTERNS ON HIV+ PATIENTS

CD4 Fluorescence Profile

Scatter	NORMAL	ABNORMAL	Total
NORMAL	13 (23%)	5 (9%)	18 (32%)
ABNORMAL	6 (11%)	32 (57%)	38 (68%)
		Grand Total	56 (100%)

The analysis of light-scatter diagrams and CD4 fluorescence profiles from HIV+ patients revealed abnormalities in these patterns not seen in normal donors; 23% of HIV+ patients studied had normal scatter diagrams and CD4 profiles, while 77% were abnormal for one or the other or for both. [3% (1/32) of normal donors studied were abnormal - not shown]

III.3. Cell Adhesion Molecule Study

III.3.i Effect of Heat-Aggregated Human IgG (AHGG)

With time in culture, cells increase in size and consequently in autofluorescence. This is accompanied by an increased expression of Fc receptors on the monocyte cell membrane which may bind antibody non-specifically and contribute to background. Initial experiments in flow cytometry showed that monocytes and macrophages bound most murine monoclonal antibodies, even irrelevant ones. This binding is nonspecific and presumably due to interactions with Fc receptors, since no binding was observed following pre-incubation of cells in heat-aggregated human IgG (AHGG) for 30 minutes at 4°C. We therefore routinely preincubated our cells with saturating amounts of AHGG before staining cultured cells.

Figure 12 clearly shows this effect. These cells are either pre-incubated (A) or not pre-incubated (B) with AHGG and stained with isotype controls which set the cursor that separates negative from positive cells. In B, the cells have bound the isotype controls nonspecifically, presumably through available Fc receptors. The cursor is set very high making subsequent analysis impossible due to the overlap of background with positive signals.

III.3.ii CD11/CD18 Expression by Growth-Factor-Stimulated Monocytes

Results of CD11a,b,c/CD18 (Fig. 13) expression were analyzed using an EPICS C (Coulter Electronics) flow cytometer. Individual single-frequency histograms were obtained with the x-axis representing fluorescence intensity (i.e. increasing antigenic density) which is divided into increasing "channels" of fluorescence (0-256 channels). Figure 13 represents an experiment from a single donor with monocytes phenotyped Day 0 (fresh) on the first row and on the other rows, cells at Day 6 in culture (unstimulated or

stimulated with GM-CSF, M-CSF or IL-3; 50Units/ml) for CD11a/CD18, CD11b/CD18, CD11c/CD18 and common β -chain (CD18). By aligning the histograms it is evident that from fresh to 6 days in culture (see fresh and Day 6 control) there is an increasing antigenic density and most cells become positive for the markers as evidenced by a shift to the right of the cursor from negative to positive. The other observation that is very clear from these histograms is that there is no significant alteration in surface antigenic density between stimulated and unstimulated cultures.

Figure 14 is a similar experiment except only one time-point (Day 7) and one marker (CD11c/CD18, the marker most prevalent on mature monocytes) are represented. Again there is no difference in expression with stimulation ; the histograms virtually overlap perfectly.

Using the same culture and staining procedures but a different flow cytometer because it has better sensitivity, the FACScan (Becton Dickinson), the experiments were repeated using the Consort-30 software for analysis, and comparable results were obtained. There was a shift towards most cells being positive for the markers, and the shift was independent of the growth factors used and at the concentrations applied.

In conclusion, the CD11 family of integrins increase on monocytes during maturation to macrophages and all cells become positive. Hemopoietic growth factors did not have an effect on these changes.

Figure 12: Effect of Human Heat-Aggregated IgG on Background Staining. Effect of pre-incubating cells with heat-aggregated IgG (also known as Gammamune) (A) or not pre-incubating (B) on background staining by isotype controls. Pre-incubation of cells (U937 monocytoid cell line) with heat-aggregated IgG at 4°C for 30 minutes binds available Fc receptors and decreases nonspecific binding by isotype controls; this keeps cursor settings low so subsequent positivity can be detected.

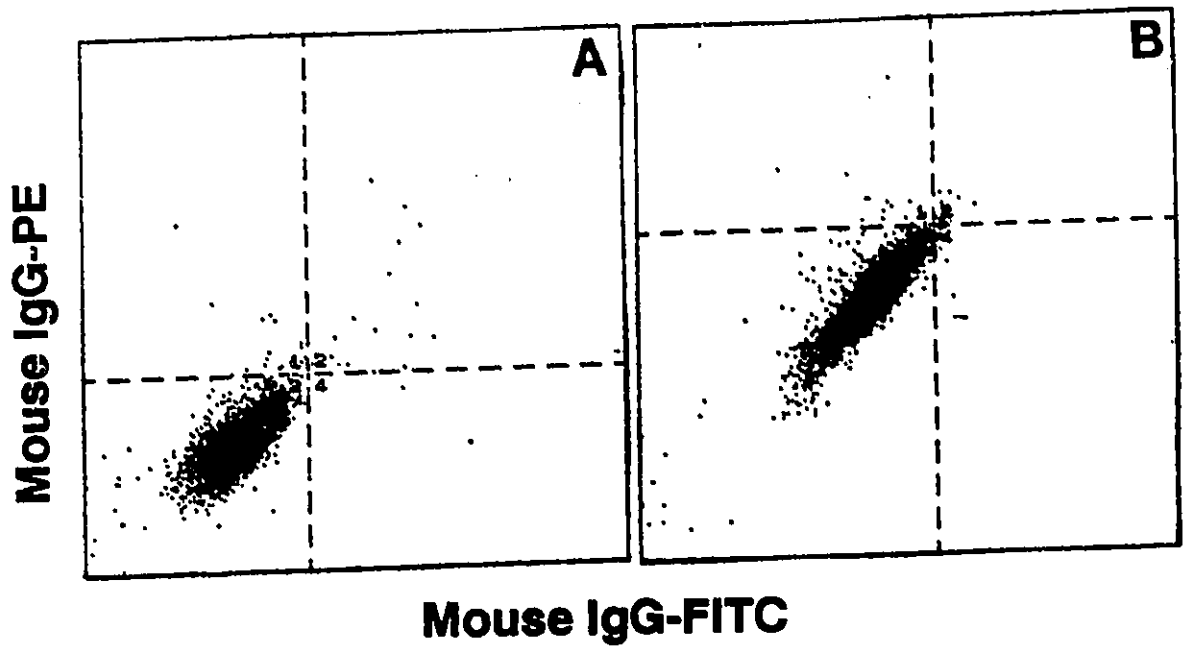


Figure 13: CD11/CD18 Group Surface Expression on Growth-Factor-Stimulated Adherent Cells. Adherent cells from human mononuclear cells were stained using indirect immunofluorescence for the CD11/CD18 group fresh and after six days in culture either unstimulated or stimulated with 50Units/ml of Growth Factors. Fluorescence intensity represents the X-axis and cell number represents the Y-axis. Maturation from monocyte (fresh) to macrophage (Day 6) is associated with an increase in the antigenic density of the CD11/CD18 integrin molecules and their common β chain. The increase appears to be growth-factor independent. Data was analyzed using an EPICS C (Coulter Electronics) flow cytometer. GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor), M-CSF (Macrophage-CSF), and IL-3 (Interleukin 3) were the growth factors used.

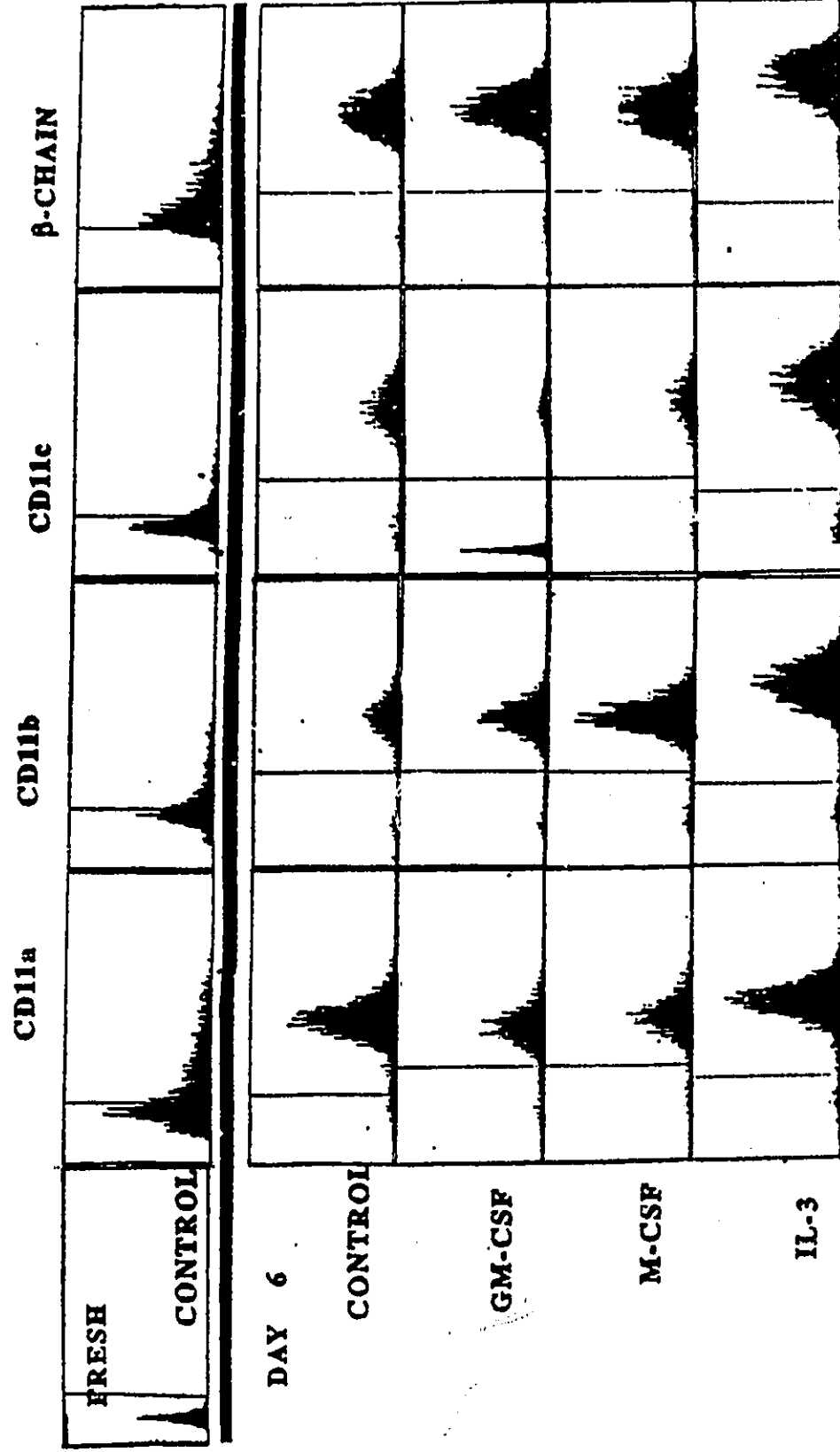
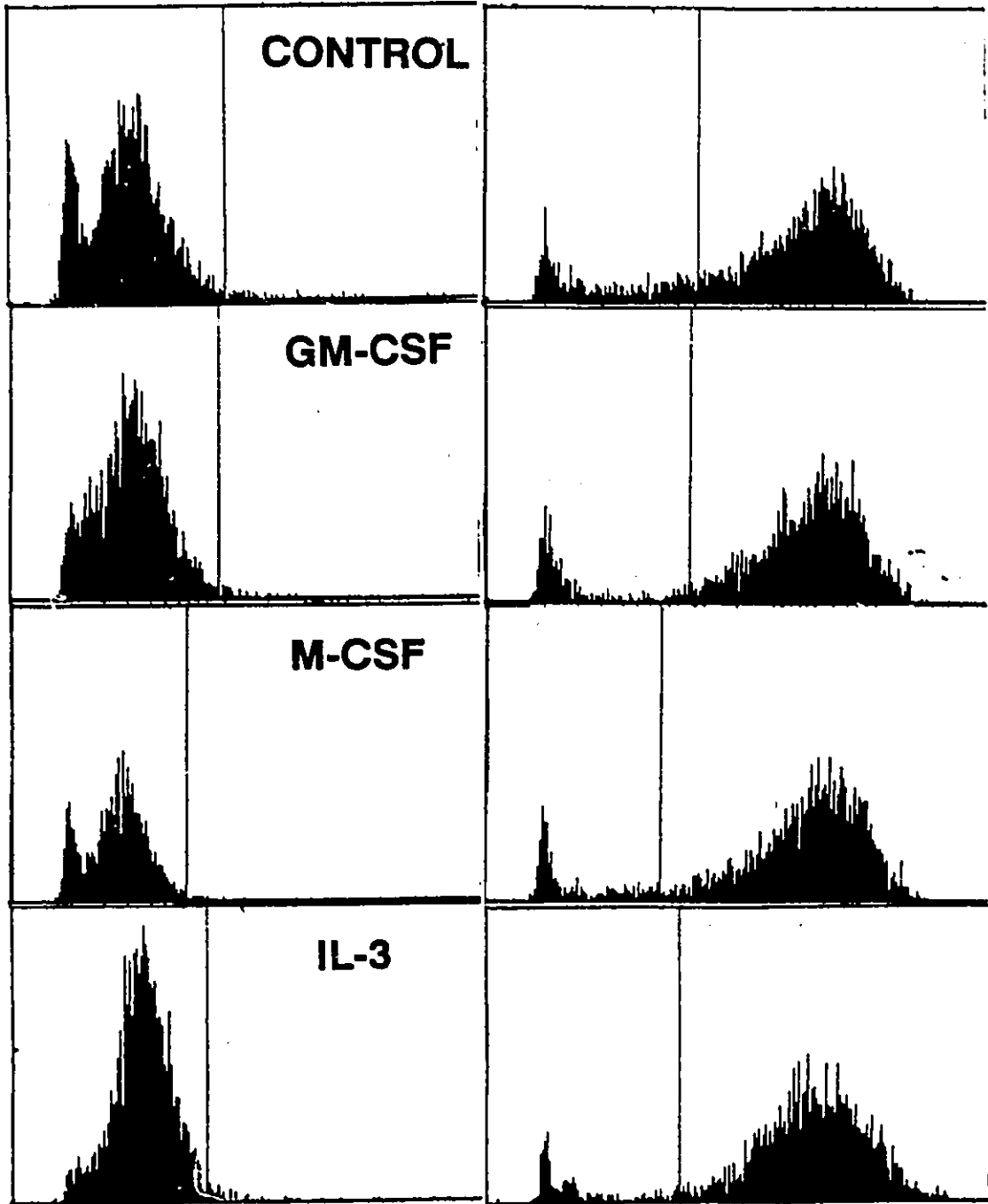


Figure 14: CD11c/CD18 Surface Expression on Day 7 Growth-Factor-Stimulated Adherent Cells. Adherent cells from human mononuclear cells were stained using indirect immunofluorescence for the CD11c/CD18 antigen (most abundantly found on monocytes) after 7 days in culture either stimulated with 50Units/ml of Growth Factor or unstimulated (control). There is no difference in fluorescence intensity with stimulation. Fluorescence intensity represents the X-axis and cell number represents the Y-axis. Data was analyzed using an EPICS C (Coulter Electronics) flow cytometer. GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor), M-CSF (Macrophage-CSF), IL-3 (Interleukin 3) were used.

CONTROL

CD11c/CD18 Positive



IV. DISCUSSION

We have taken two approaches to the study of the mononuclear phagocyte system in normal people and in patients with HIV-1 infection. Both approaches have made extensive use of immunophenotyping by flow cytometry. Early in the work it became evident that to accomplish the objectives we would have to do an extensive study of quality control issues in flow cytometry to ensure that the results would be reliable and reproducible. Thus, the study of quality control issues in flow cytometry (e.g. instrument alignment and calibration, antibodies, staining methods and analysis) became the initial practical objective. When the quality control issues had been resolved, we tackled the other objectives which were (1) to study CD4 expression by fresh blood monocytes as well as by T cells in normal and HIV-infected patients, and (2) to study the effect of growth factors on cell adhesion molecules (the β_2 family, CD11/CD18) in cultured blood monocytes. Briefly, we found that contrary to what has been reported by other investigators, all monocytes are CD4 positive and that in HIV-1 infected patients the fluorescence intensity of CD4 on monocytes is higher than in monocytes from non-infected patients, suggesting up-modulation or a change in CD4 affinity by HIV of CD4 in monocytes. In contrast, the CD4+ T cells in HIV-1 infected patients are associated with a concomitant down-modulation of CD4 receptors which appears to occur on all CD4+ T cells. This is surprising because our data suggests CD4 is down modulated in infected and in non-infected cells. By the highest available estimate, only 1 in 100 CD4+ T cells carries proviral DNA. We also found that the surface expression of the CD11 family of cell adhesion molecules increases as the monocyte matures to become a macrophage. The increase in CD11/CD18 antigen density is not modulated by M-CSF, GM-CSF or IL-3.

IV.1. Quality Control Issues in Flow Cytometry

IV.1.i Control Of Instrument Performance:

A wealth of potential information obtained from flow cytometers has been lost, or at best, greatly under-utilized because a convenient method of calibration and quality assurance has been unavailable. To ensure good, reliable, and reproducible data, proper standards and controls must be part of the staining protocol. Standards are separate materials that confirm the assay or machine is doing what it is supposed to do. Controls are similar to the samples to be measured, which reveal whether the sample preparation has been done properly, is reproducible and is what is expected. The purpose in quantitation is to get data that is independent of the instrument; variability will be inherent due to differences in amplifiers, filters, electronics, optics, etc. unless an independent standard is used which has similar characteristics to the samples. Only in this way can the effects of the instrument be minimized.

It is important to be able to determine the separation between negative and positive staining of the sample, and the relationship between the two. The lowest threshold of fluorescence the machine can measure must be known. In nonspecific staining, there is some fluorescence estimated to be comparable to 200-300 molecules of antibody (Schwartz A, personal communication). The major fluorescence is autofluorescence which is inherent in every cell sample due primarily to riboflavins (Benson et al, 1979), and is comparable to 700-800 molecules of antibody (Schwartz A, personal communication). This is real fluorescence relative to the "noise" level. Instrument "noise" is a function of the optical components (laser, filters, lenses) and electronic components (PMT, amplifiers) noise ; anything in the optical path may contribute to noise. To determine the noise level we use blank microbeads (part of the Quickcal Calibration System, see Materials and Methods and Appendix II) that have no

fluorescence of their own. Their only function is to act as a trigger so the machine will measure any noise signal in that PMT channel.

It is important to know if what is being measured is real fluorescence or noise. If the machine is very sensitive, i.e. with a low fluorescence threshold, the blank beads will fall below the autofluorescence of cells. The "window" of fluorescence the instrument is set to "see" is very important. It is usually set with the autofluorescent cells present on the fluorescence scale, and the blank beads to the left of them and not seen. This shows that what is being measured is real fluorescence. If the noise (optical and electronic) is higher than the autofluorescence, it can influence the positive counts. If the autofluorescence falls with the blank beads, there may be insufficient sensitivity of the instrument.

Noise changes with the condition of the instrument. The sample and beads should maintain the same relationship. If the PMT voltage is increased, this changes the window of analysis but does not change the relationship between standards and samples, which is therefore independent of PMT voltage.

Quantitatively, calibration plots can be prepared to get an idea of the instrument's response and level of fluorescence threshold. As mentioned, the standards and samples must have the same spectral characteristics so comparisons can be made. To obtain consistent calibration values and to avoid the problems created by quenching, the microbead standards have been calculated in units expressed as "Molecules of Equivalent Soluble Fluorochrome" (MESF). The MESF value indicates the equivalent number of free fluorochrome molecules in solution that would have the same emission intensity as the microbead standard. These units were used only in calibration and for simplicity the results are expressed as mean channels of fluorescence rather than MESFs. This system of units is valid only when the excitation and emission spectra of the standards and unknown samples are the same. Using the Quickcal beads, a calibration line is obtained and the y-intercept (a MESF value) indicates the fluorescence threshold which indicates

the fluorescence noise level of the instrument. The fluorescence threshold is related to the sensitivity of the instrument. Our instrument is calibrated daily using the Quickeal system and the sensitivity is in the 500 MESF range which is well below autofluorescence and is considered as excellent sensitivity (<1000 MESF).

IV.1.ii Cell Staining

We have standardized the procedure for staining whole blood samples from normal and HIV+ patients, with special emphasis on monocyte phenotypic analysis. A detailed protocol is included in the Methods (Appendix I - Sample Staining, Appendix II - Instrument Calibration, Appendix III - Analysis of List-Mode Data). The most practical aspects are antibody titration, selection of fluorochrome, control of background and methods of analyzing list-mode data. Briefly, it was determined that antibody titration is useful to keep nonspecific staining to a minimum; the best separation is achieved by using monoclonal antibodies conjugated to phycoerythrin because it gives off a bright signal. Cultured cells must first be pre-incubated with heat-aggregated gamma globulin to block non-specific binding due to Fc receptors. Because of the technical nature of these observations, our studies have been consolidated in a protocol for staining and analysis (Appendices I, II and III). Specific aspects will be discussed within the context of the other objectives.

IV.2. CD4 Measurement

IV.2.i Monocyte CD4

We have found that essentially all monocytes in normal and HIV+ patients are CD4 positive ($97 \pm 2.3\%$, $98 \pm 2.2\%$ respectively) and that there is no statistically significant difference in monocyte CD4 counts in persons with HIV-1 infection. This

data contrasts directly with that of Lucey et al (1991). They report that 23% of total monocytes are CD4+, implying that CD4+ monocytes are a subset of total monocytes. They also report that CD4+ monocyte counts increase during early HIV infection and then decline as CD4+ T cells decrease during progressive HIV disease. Close examination of their methods revealed that they used a similar method of staining to ours, i.e. whole blood direct two-color immunofluorescence using anti-CD14 and anti-CD4. It is unclear in their staining protocol for CD4+ monocytes, whether they used CD4 conjugated to FITC or PE, as both are listed in their methods. However, it is possible they used CD4 conjugated to FITC because in the present investigation we found that, when gating on monocytes stained with anti-CD4-FITC, on the average 32% of the monocytes are CD4 positive which is consistent with their reported number of CD4 positive monocytes. It is conceivable that the antibody affinity towards CD4 is altered with conjugation with the fluorochrome. Haas et al (1987) also used flow cytometric analysis of CD4 on CD14+ cells from fresh blood of normal donors and HIV+ patients. They performed indirect immunofluorescence using monoclonal antibodies from a source not commonly used and they reported that 53% \pm 13 (n=12) CD14+ cells were CD4+ and that there was no difference with HIV+ patients (the secondary antibody was conjugated to phycoerythrin). We also found no difference between normal donors and HIV+ patients, however in our study we found that all monocytes in both normals and patients were positive for surface CD4. Absolute counts were not available in our study and hence it is not possible to determine if there was any reduction in the absolute number of monocytes (identified by LeuM3 i.e. CD14). We can only say that the relative concentration of monocytes was normal and that the relative number of CD4+ monocytes in HIV-infected patients was not different from normal donors.

We have also observed that in HIV patients the antigenic surface density for CD4 (deduced from the mean fluorescence channel number) appears to be increased in HIV+ patients; this is significant ($p < 0.05$) when compared to normal donor values [507 ± 35

and 437 ± 11 respectively (log values), 95 ± 25 and 52 ± 5 respectively (converted linear values)]. There was no correlation between surface CD4 expression and disease progression (CD4+ T cell). Our results suggest that monocyte fluorescence became almost twice as bright for CD4 with HIV infection when looking at the linear values. This suggests an increase in antigen expression on the monocyte cell surface or alternatively a change in affinity from the normal to the diseased condition. However, there may be other extraneous factors that may have contributed to this observed phenomenon. (1) The first possibility is that there could be a CD4 conformational change that enhances the anti-CD4 - CD4 receptor interaction. The affinity may increase with infection. Recent studies at the Federal Center for AIDS indicate that monocytes are not infected in approximately 80% of patients. In our study, 86% of the patients showed that the CD4 fluorescence intensity in monocytes was higher than in normal controls. Thus, it is unlikely that the increased CD4 expression is due to HIV-1 infection of blood monocytes. It may be due to a secondary effect of the infection perhaps mediated by cytokines released due to infection. (2) Viral protein binding to the cell surface (and to CD4) may displace proteins adjacent to CD4 that may disrupt the fluorescence energy transfer that normally occurs. In the normal situation, adjacent proteins rich in aromatic amino acids (tryptophan, tyrosine) absorb the fluorochrome (conjugated to monoclonal antibody) emission wavelengths that contribute to a fluorescence quenching effect. If these proteins are not exposed to the emission they will not absorb it and the fluorescence intensity detected by the photomultiplier tubes of the flow cytometer will be increased. (3) Another possibility is that another molecule with lower affinity (e.g. Fc receptor) binds the anti-CD4 antibody and contributes to nonspecific binding. This molecule's expression may be induced with HIV infection.

To confirm our results additional studies are required; for example, it is necessary to use another independent method to measure CD4 antigenic density in monocytes (cellular radio-immuno assay). Does the increased surface expression correlate with

increased production (increased CD4 mRNA)? Perhaps there is not an increased production but rather a redistribution of intracellular CD4.

IV.2.ii T Cell CD4

Our results show a correlation ($p < 0.03$) between CD4 antigenic density and relative CD4 counts, which is an indicator of disease progression (Fig. 10). The reduction in T cell surface expression of CD4 with HIV-1 infection of cell lines has been reported previously (Hoxie et al, 1986; Klatzmann et al, 1984; Stevenson et al, 1987). It is important to note, however, that the results presented in this thesis are the first to date looking at fluorescence intensity on actual human clinical HIV+ fresh blood samples and not cell lines. We have found the surface CD4 density falls with a decline in the number of CD4+ T lymphocytes which is a hallmark of AIDS. It is understandable that depletion of CD4 from the surface of lymphocytes would result in loss of adhesion and signalling functions of CD4, interfering with the normal immune response.

The molecular basis for this receptor down-modulation is unclear. It may be by a transcriptional modulation of receptor gene expression or post-transcriptional regulation of receptor mRNA, protein processing, or by some other mechanism. Stevenson et al (1987) observed a down-modulation in HIV-infected T cell lines of CD4, CD8, CD3, CD2 and CD25. They demonstrated that HIV-infected T lymphocytes in a noncytopathic state down-regulated several cell surface polypeptides belonging to the immunoglobulin supergene family present on the T cell surface (e.g. CD2, CD3, CD4, CD8 and CD9). They could not describe the mechanism of the down-regulation since the amount of the receptors' mRNA did not appear to change significantly at any point after infection. Their data was based on HIV-infected cells over a 1 to 2 week period.

In contrast to their work, Hoxie et al (1986) demonstrated that chronically infected cells show greatly decreased amounts of CD4 mRNA. The virus strain and time-

frame were different in both groups, possibly contributing to the conflicting results. Both authors however suggested modulation of CD4 expression was due to intracellular complexes formed between viral envelope glycoproteins and the CD4 receptor molecules, which was confirmed by Crise et al (1990). Retroviral envelope-receptor complexes may thus participate in a general mechanism by which receptors for retroviruses are down-modulated and alterations in cellular function develop after infection.

Crise et al (1990) reported through studies employing co-precipitation and analysis of oligosaccharide processing and immunocytochemistry that newly synthesized CD4 and gp160 form a complex prior to transport from the endoplasmic reticulum (ER) and are retained there due to inefficient transport of gp160 ; CD4 expressed by itself is transported efficiently from the ER to the cell surface. They suggested that retention of CD4 in the ER by gp160 may partially explain the down-regulation of CD4 in HIV-1 infected T cells. The proposal by Crise et al (1990) would assume only infected T cells down-modulate, which according to our data is not the case. Other groups (Evans et al, 1988; Kong et al, 1988; Cheng-Mayer et al, 1989) using various virus isolates observed high levels of virus replication in CD4+ lymphocytes without down-modulating CD4. These observations may hold true when using cultured cell lines and plasmid constructs expressing CD4 and gp160, but the controlled *in-vitro* phenomenon may be irrelevant to the *in-vivo* situation.

It has been previously reported that 1/100,000 - 1/10,000 of peripheral blood mononuclear cells express viral mRNA (Harper et al, 1986). The study used *in-situ* hybridization to detect viral RNA and immunofluorescence to detect viral antigens. These methodologies are incapable of detecting cells that may be latently infected and not expressing viral RNA or protein. Schnittman et al (1989) used the gene amplification technique [polymerase chain reaction (PCR)] which can detect and determine the presence of cells containing either latent or replicating HIV-1. By using cell-sorting

techniques and PCR on patients' blood late in the course of AIDS, they determined that at least 1/100 CD4+ T cells contained HIV-1 DNA which is a viral burden at least 100-fold greater than previously reported. The frequency in seropositive but asymptomatic patients was determined to be in the order of 1/10,000. The high level of infection in late-stage AIDS may be the primary cause for the relentless and often accelerated decline in CD4 cell numbers and function in patients with AIDS. The data by Lewis et al (1990) support those by Schnittman. They found that by *in-situ* hybridization and confocal laser scanning microscopy, 1/350 peripheral blood mononuclear cells (PBMC) were HIV RNA-producing cells. Similarly, Ho et al (1989) reported 1/400 PBMC were HIV infected by measuring p24 core antigen in end-point-dilution cultures of PBMC.

With respect to observations presented in figure 11 and 15, if a maximum of 1/100 CD4+ T cells are actually infected with the virus, then why do all the CD4+ T cells have low density expression if they clearly are not all infected? Two populations (infected-low density, uninfected-normal) should be visible on the fluorescence profiles and dot plots. Tight single populations on the Quadstat dot plot and sharply defined peaks on the fluorescence profile are observed (fig. 12). The only difference is the position of the population on the fluorescence axis. Infected cells may release a soluble factor that affects uninfected cells and causes down-modulation of their CD4 receptor.

To confirm our observations more studies are required. For example we need to confirm the decreased expression by other methods, to determine the levels of CD4 mRNA, and to search for the conditions that promote down-modulation of surface CD4.

IV.3. Monocyte Susceptibility to HIV Infection

The next logical question is why are CD4+ T cells infected with the virus in peripheral blood and not CD4+ monocytes. Recent work by Kim et al (1990) showed that CD4 is not a limiting factor for viral growth; through transfection assays into T and

monocyte cell lines, it is seen that the long terminal repeat (LTR) of the virus genome is used efficiently by both cell types. By assaying for viral RNA content, they determined HIV-RNA expression in monocytes appears to be controlled in the same sequential manner as in T cells. This suggests that differential control of RNA expression may not be a major factor determining viral tropism. By working backward through the virus' life cycle, they determined that viral tropism is determined during the early stages of the virus' life cycle. Viral entry appears to be the major factor determining viral growth. Their observations indicate that the process of viral entry is the result of complex interactions between host and viral factors in addition to CD4 and gp120 molecules. Some of the other potential factors are outlined below:

- (1) The CD4 molecule on monocytes may not be the same as the CD4 on T cells ; the mRNA sequence may be slightly different.
- (2) The monocyte CD4 may be an isoform - it may have a post-translational modification, perhaps a glycosylation. It has been shown by Matthews et al (1987) that removal of the carbohydrate chains by enzymatic treatment from the virus's envelope gp120 proteins, blocks cell fusion with CD4 by a factor of about 50. The modification in monocytes may involve a critical area on the CD4 molecule that prevents binding.
- (3) A second molecule or factor may be needed for the virus to interact with CD4 which may be activated by virus binding and allow virus entry, or the HIV env (envelope) product may have to interact with a second cellular protein after binding to CD4. This molecule may be absent or expressed at low levels on or in monocytes. It has been reported that anti-CD4 antibodies partially inhibited HIV binding to monocytes, and anti-CD13 and anti-CD14 monoclonal antibodies almost completely inhibited binding and infection of monocytes in a dose-dependent manner (Nicholson et al, 1986). Such additional host component(s) may react differently with viral strains showing different tropisms.

(4) Recent reports have shown that CD4 on T cells is physically associated with the cytoplasmic protein tyrosine kinase p56^{lck} (Shaw et al, 1989); this raises the possibility that CD4 can modulate the protein kinase activity of p56^{lck} which could play a role in T cell activation. This may be the signal necessary to permit viral entry into T cells. To date it is not known if the CD4 on monocytes is associated with a protein kinase. If it is absent, the activating signal necessary for cell activation and viral entry may be missing.

(5) It seems very likely that simple positioning of CD4 on the monocyte ruffled membrane may be the cause of inefficient virus binding and infection. The OKT4 antibody (CD4 specific, Ortho Diagnostics) recognizes the CD4 molecule on T cells but not on monocytes indicating the OKT4 binding region is not accessible on monocytes. Similarly, the virus may bind but efficient gp120-CD4 interactions are blocked, and therefore so is virus entry and infection.

(6) Retroviruses conventionally require cell replication for infection. Monocytes are considered to be in a state of transition while circulating in peripheral blood before differentiating to specific tissue macrophages and are not considered to have much proliferative potential. The most sensitive detection method for virus DNA is PCR whose sensitivity is between 3 to six copies of DNA. Since the monocyte is not a proliferative cell, viral replication may not reach detectable levels. Thus, even if they are infected, the number of copies may be below the sensitivity of the assay during their brief time in the circulation. Coupled with the fact CD4 is expressed at low surface levels and that the monocyte is a relatively non-reactive cell may explain its resistance to infection.

(7) As previously described in the introduction, tissue macrophages are uniquely differentiated end-stage cells that are part of the myeloid lineage and are directly derived from circulating monocytes. There have been many studies proving that virus could be cultured from peripheral blood monocytes (Ho et al, 1986; Popovic and Gartner, 1987) and that various tissue macrophages are infectable (Gendelman et al, 1988; Koenig et al, 1986, Clarke et al, 1990). Somehow in their transition between the endothelial barrier

they acquire characteristics that render them susceptible to infection. It has been reported that macrophages increase their expression of Fc receptors (specifically, FcRIII, Klaassen et al, 1990). It is conceivable that HIV can infect some cells by binding to antibodies that do not neutralize it. This could actually facilitate virus entry into macrophages through an interaction of a portion of the antibody (Fc portion) with either a cellular Fc or complement receptor. Many reports of infected cultured monocytes require in-vitro maturation and/or treatment with colony stimulating factors before infection occurs.

As previously mentioned, the up-modulation of CD4 on the monocyte surface appears to be a secondary effect of HIV-1 infection. The mechanism responsible for the increase of surface CD4 as well as the significance of this finding is unknown. We should also mention that the possible physiological role of both surface and intracellular CD4 in monocytes is unknown. Our observations may provide a lead into the investigation of the functional role of CD4 in monocytes and macrophages.

IV.4. Cell Adhesion Molecule Study

Our study of the CD11/CD18 family in monocytes in culture has clearly shown that all members of the family increase their surface expression and antigenic density. Essentially all cells are positive at the end of the culture period. The addition of growth factors (M-CSF, GM-CSF and IL-3) did not have an effect on the expression of the CD11 markers. Our findings suggest that expression of CD11 markers by macrophages is perhaps genetically programmed and not susceptible to modulation, at least not by the growth factors used. Due to the limited scope of our study of the CD11 family we can only speculate that the expression of CD11 plays an important role in the cellular communication of macrophages with their surrounding environment as well as with other cells.

V. SUMMARY

1. Our results have shown that the measurement of fluorescence intensity for CD4 in monocytes may be an additional parameter for the study of this disease. However the significance of this measurement remains to be determined .
2. The controversy of how many monocytes are CD4+ has been resolved; virtually all monocytes are CD4+. A staining system was developed incorporating instrument alignment, staining and analysis so that reproducible and reliable results could be obtained. This work can help in setting up quality control procedures in flow labs so data from different instruments, operators and labs can be compared. (Some of this work has been used in setting up a proposal for a consensus in quality control procedures for flow cytometry labs participating in a National Clinical Trials Network involved in assessing HIV therapies.)
3. Our observation that the antigenic density of CD4 positive T cells has decreased in patients with HIV-1 infection also needs to be studied further to establish the mechanism of down-modulation as well as the immunological significance of low CD4 expression.

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VII. APPENDICES

Appendix I

SAMPLE STAINING

All patient (HIV+) and normal donor fresh blood samples were prepared in an identical manner as follows:

1. The samples were collected in EDTA vacuum collection tubes by venipuncture.
2. 100µl of whole blood was aliquoted from each blood sample into a 12 x 75mm test tube.
3. Appropriate amounts of each monoclonal antibody were aliquoted to the test tube. Two- color immunofluorescence was usually done using directly conjugated monoclonal antibodies from Becton-Dickinson
The standard antibody panel was:
 - i. IgG1-FITC (10µl)/IgG1-PE (10µl) (Isotype controls)
 - ii. Leu3a-PE (10µl)/LeuM3-FITC(10µl) (CD4/CD14)
 - iii. Leu2a-PE (20µl)/Leu4-FITC (20µl) (CD8/CD3)
 - iv. Leu3a-PE (20µl)/Leu4-FITC (20µl) (CD4/CD3)
4. Samples were incubated for 10-15 minutes at room temperature and then placed into an automated erythrocyte lysing instrument (Q-Prep, Coulter Electronics) which lyses the erythrocytes, adds a stabilizer (PBS) and fixes the cells (0.5% para-formaldehyde) automatically, all in the original test tube.
5. The test tubes were taken out of the unit and washed twice with 4ml of PBS (1500 rpm, 5 minutes).
6. After the final wash, the PBS was aspirated and 1ml of cold 2% para-formaldehyde was added drop by drop while the sample was being gently vortexed.
7. The samples were either analysed by the FACScan flow cytometer or put into the dark in the fridge (4°C) and acquired within 24 hours. The acquired information from the FACScan was saved onto computer disks (3.5") so data analysis could be done at a later time. [*Samples from Dr. Diaz's patients (analyzed post-QC3 implementation) were all run on the flow cytometer within an hour after staining to minimize light exposure and lag time between staining and acquiring]

Appendix II

DAILY INSTRUMENT CALIBRATION PROTOCOL for the FACScan FLOW CYTOMETER (Becton-Dickinson)

PART I: Autocompensation

Two test tubes are prepared with CaliBRITE Beads (Becton-Dicknson) as follows:

- A. (i) Unlabelled bead suspension: One drop of unlabelled bead stock solution is added to 1ml filtered isoton
(This tube is used for photomultiplier tube (PMT) detector adjustment)
- (ii) Labelled bead suspension: One drop of each labelled bead stock (FITC stock and PE stock) is added to 3ml of filtered isotone
(This tube is used for fluorescence compensation and sensitivity testing)
- B. The AutoCOMP Software is run and all printouts with labelled run numbers corresponding to the QC3 run numbers assigned are retained and recorded daily. The lot numbers and expiration date are kept on file with the AutoCOMP print-outs.

*All reagents are stored at 2°C. The two test tube suspensions can be used for a one week interval provided they are kept refrigerated and protected from light.

PART II: Preparation of Representative Sample and Target Channelling

A. Representative Sample

Double-stain normal whole blood with isotypic FITC and PE controls and Leu3a-PE (CD4) and Leu2a-FITC (CD8) (SAMPLE 1; representative sample) as follows:

- appropriate amounts of antibody (as specified by manufacturer) are incubated with 100µl of human venous whole blood for 10 minutes at room temperature after brief vortexing.
- sample is processed in Q-Prep unit (Coulter Electronics) using the 35 second cycle
- 3ml PBS is added and sample is spun at 1500 rpm for 5 minutes at 4°C
- supernatant is aspirated and wash repeated with 4ml PBS
- supernatant is aspirated and pellet is resuspended by dropwise addition of 1ml of 2% paraformaldehyde while vortexing
- in a separate test tube, 2 drops of QC3 bead stock solution are added to 500µl of PBS and 2 drops of this solution is added to SAMPLE 1.

*A representative sample is prepared only when beginning a new lot number of QC3 beads.

B. Target Channelling

- SAMPLE 1 is acquired. (All acquisition is done using the FACScan Research Software)
- Forward scatter and side scatter is adjusted so the full profile of the blood (lymphocytes, monocytes, granulocytes) and the QC3 beads can be seen.
- The control sample is run. FL1 and FL2 PMT voltages are adjusted so the control population falls within the first log
- SAMPLE 1 is run again. The FL1 and FL2 PMT voltages are adjusted so the labelled population of each fluorescent signal appears in the middle of the intensity range
- Compensation is adjusted so that each fluorescent population is proportional to the control population in quadrant 1
- All instrument settings are noted and a run number assigned
- SAMPLE 1 is run again at these instrument settings and a print-out of the light-scatter diagram and the quadstat is obtained
- QC3 target channel positions are recorded from list-mode analysis along with their median peak channels. These median peak channels are the target channels for daily QC3 runs and are recorded on a table together with the instrument setting from the representative sample.

PART III: QuickCal™

(QuickCal™ is run daily immediately following the QC3 run)

- A fresh QuickCal sample is prepared daily by adding two drops of QuickCal stock beads (FCSC Corp) to 500µl PBS
- All instrument settings from the QC3 runs are maintained except colour compensation which is reduced to 0.00%
- The same run number is assigned to the QuickCal beads as assigned to the QC3 run.
- FACScan Research Software is exited and QuickCal Software is entered
- Data is analysed using this automated software and a print-out obtained which includes the fluorescence threshold sensitivity (expressed in MESF units), an important indicator of instrument performance. A description of the beads is found in the Materials and Methods section.

Daily QC3 Calibration

Each day a fresh QC3 preparation is prepared and run on FACScan Research Software as follows:

- One drop of QC3 stock is added to 500µl PBS
- Colour compensation and the side scatter voltage is adjusted to that recorded from the representative sample.
- The bead solution is run and the median peak channel on the single histogram of FL1 is identified
- The FL1 voltage is adjusted so that MPC is ± 2 channels from the target channel (set with the representative sample)
- The voltage adjustments are repeated for FL2 and amplifier adjustments are made for forward-scatter so they fall within two channels of their listed targets
- All instrument settings and histogram median peak channels are recorded on the table initiated with the original representative sample run.
- PMT/AMP settings are plotted daily on a graphic chart. Any major variations in instrument settings or any difficulty in reproducing median peak channels are reported and the problems resolved as soon as possible.

Appendix III

List-Mode Data Analysis

(FACScan Flow Cytometer)

Data acquired by the flow cytometer can be processed immediately into histograms and dot plots (real-time analysis) or the measurements are stored in or on disk in time sequence (list-mode). List-mode data has the advantage that the data may be reprocessed, gated, and displayed as often as required. This feature is very useful when it is not clear where to set gates and when time does not permit immediate analysis.

The minimum number of events (i.e. individual cells) acquired and analyzed by the flow cytometer was 10,000 (the actual maximum can be as high as 100,000). The data was saved to a file specified by a unit number (in this case a disk drive with a removable microdiskette), a file identification code (an alphanumeric name given to identify the project) and a file tag (a three digit extension for the file ID which automatically increments when data is saved).

When the data is finally analyzed, analysis gates are first defined to separate cell populations of interest from the rest of the sample. The analysis was performed on histogram and dot plot representation of the data. Histograms represent the frequency distribution for a single parameter where the number of events is indicated along the vertical axis and the measured intensity is represented on the horizontal axis. Dot plots represent the frequency distribution of two parameters where the measured intensity of the parameters is indicated along the horizontal and vertical axes.

We were interested in the expression of surface CD4 on monocytes and T cells. It was determined that CD4 could replace CD14 as a monocyte marker (table 6), and that both monocytes and a subset of T cells were CD4+. The usual method of analysis is to gate a single population. This gate often proved to contain contaminating T cells especially in patients' samples where T cells are activate, blast and increase in size. This changes their light-scattering properties and they move into the monocyte light-scattering region. We introduced a new gating method that would eliminate the problem of contaminating T cells present in the monocyte gate. By gating on both lymphocyte and monocyte populations (A) using the single histogram for analysis, two distinct peaks were seen in normal donors where the dimly positive CD4 peak represented CD4+ monocytes and the tall sharp peak represented the bright, high surface-expressing CD4+ T cell (B). The percentages and mean channel numbers were obtained from the statistics provided by setting the cursors between the negative and two positive peaks.

This approach proved very effective when analyzing healthy donors, but when patients were analyzed in this way it was found that 68% of the HIV+ patients presented an unusual CD4 fluorescence profile due to shifts in surface CD4 expression. This complicated our single histogram CD4 profile analysis due to peak overlap which made cursor setting difficult.

To overcome the problem with overlap often seen in patients, the gate was kept the same (to include monocytes and lymphocytes), but we used the CD3-FITC/CD4-PE combination and the dot plot (Quadstat) bivariate display. No overlap is seen because the monocytes (CD4+ only, found in Quadrant 1) and the CD4+ T cells (positive for CD3

and CD4, found in Quadrant 2) were clearly distinguishable and did not overlap, facilitating data analysis (C).

All fluorescence data is expressed in log. When calculations are performed on log data, the channel values are first converted to their corresponding linear value, the operations performed, and converted back to log channel values that range from 0 to 1024 channels. For example, if a peak has a mean fluorescence value of 512 and another has a value of 256, the first is not two times as bright, but rather 10 times as bright. In order to correctly plot the mean channel numbers which actually represent log values, the numbers were converted to linear values using the equation :

$$10^{\text{channel number}/256}$$

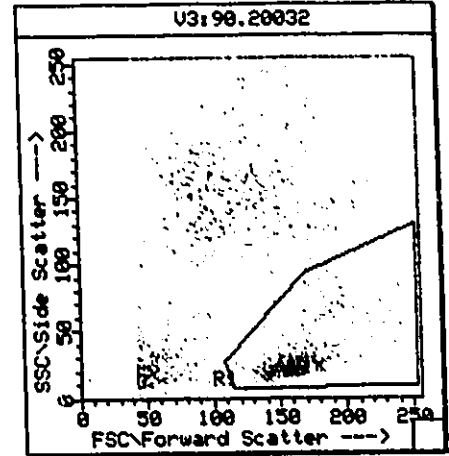
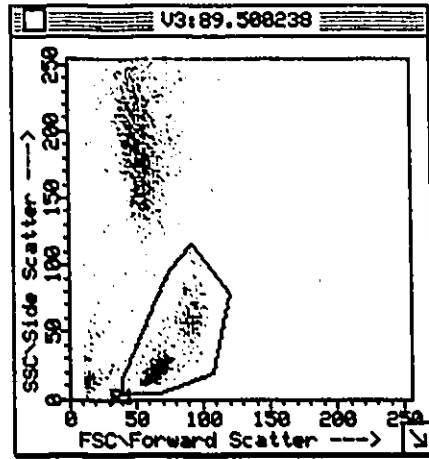
so they could be plotted on a linear scale.

Figure 15: CD4 on Human Blood Cells. This is a summary of list-mode data from normal donors and HIV+ patients. These are typical representations of their light-scatter diagrams (A), CD4-PE profiles (B), and Quadstat dot-plots (C). The HIV+ patient usually had a messy light-scatter and overlapping T cell CD4 and monocyte CD4 peaks on their CD4-PE profile. The Quadstat analysis and CD3-FITC/CD4-PE staining combination clearly differentiates the two populations.

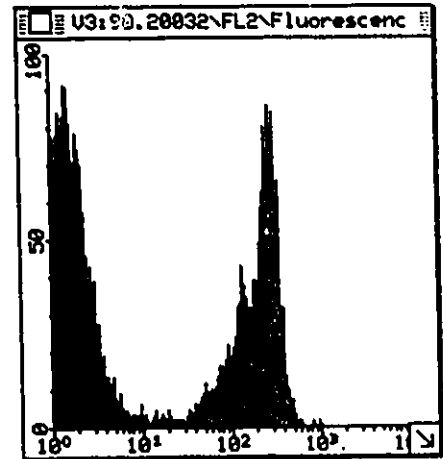
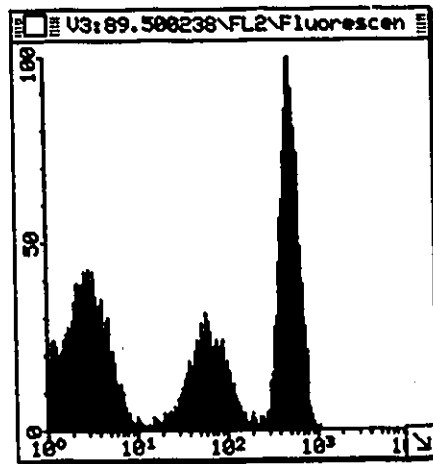
NORMAL

HIV+ PATIENT

A



B



C

