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The Role of Surrogate Markers of HIV Infection

Report prepared by: C. M. Tsoukas, M.Sc., M.D., FRCP(C) Associate Director McGill AIDS Centre Montreal, Quebec, Canada

Canada

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Health Canada
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Contributors

Dr. Jacques Bouchard (Health Canada, Ottawa)

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1. Introduction

Initial infection with Human Immunodeficiency Virus (HIV) is followed by an asymptomatic period of variable duration that is characterized by a low or absent rate of virus replication, stable or slowly decreasing numbers of helper T cells, and qualitative defects in T cell function. Generally, the progressive, slow, and irreversible destruction of the immune system is not clinically apparent for many years. For any particular individual, the likelihood and timing of the development of clinical Acquired Immunodeficiency Syndrome (AIDS) following seroconversion is not readily predictable. Owing to the variable clinical expression of HIV infection, the use of non-clinical disease markers (surrogate markers) has become critically important to patient management.

The various phases of HIV infection, including the early asymptomatic phase, are closely associated with quantifiable laboratory findings[111,112]. For example, immune dysfunction in advanced cases of the disease is clearly related to the profound depletion of helper T cells, in addition to an increase in HIV antigenemia and viremia[150].

1.1 Surrogate Markers

By definition, surrogate markers of HIV infection are measurable traits that correlate with the development of clinical AIDS. Although several candidate surrogate markers have been described, only a few have shown promise. Ideally, such markers should

1) allow patients at highest risk of disease progression to be identified.

2) aid in estimating the duration of infection.

3) assist in disease staging.

4) predict development of indicator disease (opportunistic infections of AIDS).

5) measure, *in vitro*, the therapeutic efficacy of immuno-modulating or anti-viral treatments.

In addition, surrogate markers must be
- **!** easily quantifiable,
- **!** reliable,
- **!** clinically available, and
- **!** affordable.

1.2 Depletion of CD4+ T Cells

The most characteristic feature of AIDS is a selective depletion of the CD4+ T helper/inducer subset of T cells. The degree of CD4+ T cell depletion is currently the single most important laboratory finding considered when making recommendations regarding therapy with anti-retroviral drugs. Most experimental therapeutic protocols enroll patients on the basis of CD4+ T cell counts, or the presence or absence of viral antigenemia, or both. These surrogate markers of clinical AIDS development are used to make decisions on the timing of medical intervention, to predict actuarial outcomes, and to plan future health-care expenditures.

1.3 About This Report

This report examines the current state of knowledge, the clinical usefulness, and the role of surrogate markers in the natural history and treatment of HIV infection.

The remainder of this report is divided into the following sections:

- Section 2, "*Pathophysiology*," which describes the disease phases of HIV infection.
- Section 3, "*Serologic T Cell Activation Markers*," which describes assays and status of HIV disease of markers of HIV infection, including beta$_2$-microglobulin, neopterin, and soluble interleukin-2 receptor.
- Section 4, "*Serologic B Cell Activation Markers*," which describes the usefulness of such markers as interleukin-6, immunoglobulins, and circulating immune complexes.
- Section 5, "*Antibodies to HIV*," which describes anti-p24, anti-gp120, anti-p17, anti-gp41, and anti-NEF.
- Section 6, "*Other Antibodies*," which describes other autoimmune phenomena related to HIV.
- Section 7, "*Other Serologic Markers*," which describes tumor necrosis factor, acid-labile human leukocyte interferon, and 2-5A synthetase.
- Section 8, "*Antigen Markers*," which describes the p24 antigen marker.
- Section 9, "*Cell Surface T Cell Activation Markers*," which describes the relationship between serologic activation markers and cell surface activation markers.
- Section 10, "*CD4+ T Cells*," which describes the technology, protocol, and other aspects associated with the use of CD4+ T cells as surrogate markers of HIV infection.
- Section 11, "*Discussion and Recommendations*," which describes the benefits associated with the use of surrogate markers, criteria for defining satisfactory surrogate markers, and the selection of a preferred marker.
- Section 12, "*Glossary*," provides definitions of the acronyms used throughout the report.
- Section 13, "*References*," lists all references cited throughout the report.

2. Pathophysiology

Large prospective studies on the natural history of HIV infection have provided insight into the pathophysiology of the illness[46,71,112,197]. Surrogate markers for HIV infection have been studied in two disease phases of HIV infection:

- early (immunocompetent) phase, and
- late (immunodeficient) phase.

2.1 Early (Immunocompetent) Disease Phase

Early in the infection, the numbers of leukocytes, lymphocytes, and T cells are normal. However, numbers and percentages of T cell subsets begin to change soon after seroconversion. Levels of CD8+ T cells rise dramatically upon infection with HIV and promptly return to just above the baseline[112]. Such an increase may represent cytotoxic T cells attempting to control HIV infection.

The initiation of HIV infection is characterized by immune system priming and activation of immune effector cells. As a result, T cells become activated and express increased levels of IL-2R and MHC-encoded HLA-DR antigens[155]. Soluble molecules secreted by activated T cells, and antibodies specific to the envelope and core proteins of HIV, are also found in greater concentrations in the serum of individuals recently infected with HIV[6,56].

Table 2-A lists types of markers and their mechanisms for the early (immunocompetent) disease phase of HIV infection.

Table 2-A
Markers and Mechanisms—Early (Immunocompetent) Disease Phase

Type of Marker (Activation)	Mechanism (Host-dependent)
Cellular Markers	HLA-DR+ IL-2R+ T Cells
Soluble Markers	$ß_2$-M neopterin sIL-2R sCD4 sCD8
Antibody Production	anti-gp120 anti-p24 IgA

2.2 Late (Immunodeficient) Disease Phase

The late stage of infection, immediately preceding the development of clinical AIDS, is characterized by

- changes in cytokine production,
- a marked decrease in the ability to respond to neoantigens[111], and
- a decline in numbers of CD4+ T cells[112].

Antigenemia and viremia are also found and are indicative of viral replication.

Table 2-B lists types of markers and their mechanisms for the late (immunodeficient) disease phase of HIV infection.

Table 2-B
Markers and Mechanisms—Late (Immunodeficient) Disease Phase

Type of Marker (Immune Dysfunction)	Mechanism (Virus-dependent)
Cellular Depletion	CD4+ T Cells
Cytokine Depletion	IFN IL-2
Antibody Depletion	anti-p24 anti-gp120

3. Serologic T Cell Activation Markers

Serologic T cell activation markers of HIV infection include:

- beta$_2$-microglobulin (ß$_2$-M),
- neopterin, and
- soluble Interleukin-2 Receptor (sIL-2R).

3.1 Beta$_2$-microglobulin

Beta$_2$-microglobulin is a polypeptide containing 100 amino acids (molecular weight 11.8 kDa). Together with an MHC-encoded heavy chain, ß$_2$-M forms the Class I molecules HLA-A, HLA-B, and HLA-C that are present on the surface of most nucleated cells[94]. ß$_2$-M is also present in most biologic fluids, at low concentrations. ß$_2$-M is eliminated by the kidneys, where, following glomerular filtration, it is reabsorbed and catabolized in the proximal tubular cells. Clearance is very efficient, with less than 0.1 percent of filtered ß$_2$-M excreted in the urine of healthy persons. The serum concentration of ß$_2$-M increases when glomerular filtration decreases or when the production of ß$_2$-M increases as a result of renal, neoplastic, inflammatory, or immunological disease[74]. Urinary excretion of ß$_2$-M increases following treatment with anti-neoplastic, anti-microbial, and anti-inflammatory drugs[54].

3.1.1 Assays

Beta$_2$-microglobulin can be measured in biological fluids with commercially available quantitative competitive immunoassays, such as enzyme-linked immunosorbent assays (ELISAs) or competitive radio-immunoassays. In the latter assay, the competitive capacity of test material is compared with that of standards having a known ß$_2$-M concentration.

3.1.2 Status in HIV Disease

In 1982, early in the AIDS epidemic, increased ß$_2$-M was reported in the serum of homosexual men having AIDS[22,26,72,182]. At that time, AIDS was diagnosed solely by clinical presentation, although laboratory abnormalities were used to support the diagnosis in suspected cases. The major problem that physicians faced was that of recognizing asymptomatic or subclinical disease before the manifestation of opportunistic infections or Kaposi's sarcoma. Because HIV was not yet identified as the infectious agent causing AIDS, surrogate markers of clinical disease development were of particular interest and an important area of investigation.

In one early study, elevated serum ß$_2$-M clearly identified homosexual and drug-abusing men with AIDS or suspected of having AIDS. The study suggested that quantitation of ß$_2$-M could have diagnostic value when screening high-risk groups for AIDS[215]. In two groups of homosexual men,

evaluated prospectively in 1983 and 1985, serum $ß_2$-M was elevated in 64 percent of HIV-infected individuals, but in only 6.7 percent of uninfected controls[104]. Serum $ß_2$-M levels of greater than 3.0 mg/litre in the HIV-infected group were associated with progression to AIDS.

Viral Diseases and Lymphoproliferative Disorders

High levels of $ß_2$-M have been reported in patients having various viral diseases including those infected with CMV. High levels of $ß_2$-M have also been reported in patients having lymphoproliferative disorders such as lymphomas[54]. Because these two disease states may be present in the late stage of HIV infection, it is important to interpret the data on serum $ß_2$-M levels in the context of the total clinical picture. Elevated serum $ß_2$-M has been noted in certain uninfected individuals in high-risk groups for AIDS, such as haemophiliacs and drug abusers[20,56,159,175]. In these groups, abnormally high serum $ß_2$-M levels correlated well with increased transaminases and underlying chronic hepatitis, rather than intravenous use of factor concentrates or drugs.

Longitudinal Studies of High-Risk Groups

The results from several longitudinal studies of high-risk groups have also shown that $ß_2$-M is an early marker of HIV infection. Serum concentrations above the background level are detected in most infected individuals within six months of seroconversion[11,12,26,48,49,67,75,76,77,78,92]. Infected individuals with high (or low) levels of $ß_2$-M at the end of the first year tend to remain that way for several years.

The magnitude of CD4+ T cell decline does not correlate with the rise in $ß_2$-M levels in the first year. From two to three years after seroconversion, the initially increased $ß_2$-M levels did correlate inversely with the rate of decline of CD4+ T cells[76]. An elevated level of $ß_2$-M in male homosexuals was the single most powerful predictor of progression to AIDS in a European cohort study, the Multicenter AIDS Cohort Study in Los Angeles, and in the San Francisco General Hospital Cohort Study[135].

Cerbrospinal Fluid (CSF)

The ratio of $ß_2$-M in CSF to $ß_2$-M in serum may be of some use as a marker for HIV-associated neurologic complications. It appears superior in this regard to CSF $ß_2$-M concentration alone[82]. A recent small study found $ß_2$-M in the CSF of patients having AIDS dementia complex[45].

Other Factors

Serum $ß_2$-M levels are observed to be higher in HIV-infected pregnant women than uninfected pregnant women. These levels were directly related to HIV status and not to pregnancy or month of gestation[110].

No ethnic, sex, or racial differences are noted among HIV seronegative intravenous drug abusers. However, significantly higher levels of serum $ß_2$-M are observed in HIV-infected white intravenous drug abusers than in black intravenous drug abusers, despite similar CD4+ T cell counts[208].

Zidovudine (AZT) Treatment

Patients with AIDS or AIDS-related complex (ARC) who were treated with AZT exhibited a decrease in serum $ß_2$-M after 8 to 12 weeks of treatment[86,87,88]. This decline was not sustained, however; by the sixth month after treatment, serum $ß_2$-M returned to baseline pre-treatment levels in two separate studies[86,87,88].

Usefulness of $ß_2$-M as a Marker in Infants

The usefulness of serum $ß_2$-M levels as a marker of disease progression has been studied in the early stage of vertically acquired, perinatal HIV infection. Such HIV-infected infants do not always exhibit CD4+ T cell depletion at six months of age. Most, however, have elevated serum $ß_2$-M levels. Those with the highest levels are more likely to progress to clinical AIDS[35,41,116,117,203,204].

3.2 Neopterin

Neopterin (6-D-erethro-trihydroxpropylpterin) is a compound of low molecular weight derived from dihydroneopterin triphosphate. Neopterin is an intermediate in the synthesis of tetrahydrobiopterin from GTP. Before the AIDS epidemic, high neopterin levels in serum or urine were found only in patients with atypical phenylketonuria (a congenital deficiency of phenylalanine hydroxylation)[44].

Elevated neopterin levels have also been reported in conditions that involve rapid cellular turnover and activation of the immune system[81,141]. Patients who receive biological response modifiers, such as IFN-α, IFN-τ, IL-2, or TNFα exhibit high serum neopterin levels. Monocytes and macrophages, in particular those stimulated with IFN-τ, are an important source of neopterin[81]. Neopterin production increases in numerous infectious and inflammatory disorders including viral, bacterial, and fungal infections, aseptic meningoencephalitis, kidney graft rejection, acute graft-versus-host

disease, collagen vascular diseases, and the advanced stages of certain malignancies[80,81,141,142].

The function of neopterin is unknown.

3.2.1 Assays

Neopterin is present in both urine and serum[211]. Reverse-phase HPLC is used to determine the concentration of neopterin in urine[194]. A commercially available radio-immunoassay kit is available for measuring levels of neopterin in serum, plasma, and CSF[61].

3.2.2 Status in HIV Infection

Neopterin as an Early Marker

Based on urine and serum neopterin measurements, AIDS and ARC patients can easily be distinguished from seronegative individuals[1,8,17,18,32,48,49,57,58,59, 60,62,63,176]. Urine and serum neopterin is also elevated in patients having HIV-related persistent generalized lymphadenopathy and in asymptomatic HIV-infected individuals. Therefore, elevated urine or serum neopterin appears to be a very early marker of HIV infection[98,107,108,162,163].

Neopterin and Disease Progression

Longitudinal studies have revealed that neopterin levels correlate with disease progression[62,99]. The Multicentre AIDS Cohort Study reported serum neopterin levels to be the strongest predictor of the progression of HIV infection to clinical AIDS. Not only did serum neopterin levels predict disease progression, but they also predicted the rate of CD4+ T cell decline as early as three years in advance[126].

Blood and CSF

Neopterin levels in blood and CSF are also elevated in patients having HIV-associated neurologic complications. This finding may reflect the activation of macrophages within the central nervous system[184].

Children and Pregnant Women

The predictive value of neopterin measurements in seropositive pregnant women or in children with perinatally acquired HIV infection is unclear.

3.3 Soluble Interleukin-2 Receptor

Soluble IL-2 Receptor (sIL-2R) is the secreted form of the IL-2 Receptor. High-affinity IL-2R is composed of two different IL-2-binding peptides: IL-2Rα (molecular weight 55 kDa) and IL-2Rß (molecular weight 75 kDa). It has been observed that following the activation of T cells by antigens or mitogens, IL-2Rα (also known as Tac peptide) is released into the supernatant of *in vitro* cultured cells. Elevated serum levels of IL-2Rα are also detected *in vivo* in clinical conditions associated with lymphocyte activation, such as Adult T cell and Hairy cell leukemias, acute and chronic lymphocytic leukemias, rheumatoid arthritis, systemic lupus erythematosus, and sarcoid and multiple sclerosis[168].

3.3.1 Assays

sIL-2R can be measured in serum using a fluorescent sandwich ELISA[173] or a competitive radio-immunoassay[192].

3.3.2 Status in HIV Disease

IL-2 (or T cell growth factor) is a lymphokine that is generated by mitogen- or antigen-activated T cells. This lymphokine interacts with specific high-affinity IL-2R on the surface of other activated T cells. sIL-2R is derived from such activated T cells. Because T cell activation may amplify HIV replication, identification of individuals with high levels of sIL-2R may be an important prognosis indicator[79,84].

In one study, 50 percent of seropositive blood donors had increased levels of sIL-2R when compared with seronegative controls[155]. In this study, an inverse relationship was noted between sIL-2R levels and CD4+ T cell counts.

In a second study, 73 percent of patients with AIDS, 80 percent of those with ARC, and from 75 to 85 percent of asymptomatic HIV-infected individuals had elevated sIL-2R levels[96]. Soluble IL-2R levels reflected AIDS CDC classification status and correlated negatively with CD4+ T cell numbers, lymphocyte count, and CD4+/CD8+ T cell ratios. However, sIL-2R levels failed to correlate negatively with leukocyte or B cell counts[79]. The highest levels of sIL-2R were found in patients with CDC group IV_D disease (1,006 ± 289µ/ml). Significant differences were noted between CDC classification groupings as well as between asymptomatic HIV-infected individuals and uninfected individuals (210 ± 149 µ/ml versus 74 ± 24 µ/ml; $P < 0.001$). In this study, it appeared that elevated sIL-2R levels in AIDS patients were not the result of secretion of the receptor from cells, but rather were the result of destruction of either activated T cells or CD4+ T cells.

Patients with HTLV-1-associated T cell leukemia have increased serum levels of sIL-2R. Quantitation of this marker is useful for diagnosis and response to chemotherapy. In HIV-infected haemophilia patients, elevated sIL-2R levels may be associated with EBV or CMV infection, rather than a stage of HIV infection[138]. Care must be taken when interpreting serologic findings in this group.

Whether sIL-2R measurement will become useful in predicting disease progression remains to be determined. Large cohort studies of the usefulness of sIL-2R quantitation in biological fluids as a surrogate marker for development of clinical AIDS have yet to be carried out. Although levels of sIL-2R are associated with immune system activation, measuring this marker is more difficult than measuring other serologic markers. In addition, assay systems for sIL-2R are not widely available.

4. Serologic B Cell Activation Markers

4.1 Interleukin-6

HIV-infected individuals exhibit B cell activation and hypergammaglobulinemia. IL-6, a B cell stimulatory factor, is elevated in the plasma of HIV-infected patients[137]. No correlation has been observed between IL-6 levels and HIV disease status or the presence of opportunistic infections. IL-6 is produced by Kaposi's sarcoma cells and may act as a growth factor for them[121]. Information with regard to the prognostic value of this marker in the development of clinical AIDS is unknown.

4.2 Immunoglobulins

In AIDS patients, functional abnormalities of B cells, including polyclonal B cell activation, hypergammaglobulinemia, raised titres of antibodies to various pathogens, and autoantigens were documented early in the AIDS epidemic[3]. Serum immunoglobulin (Ig) concentrations also increase in asymptomatic HIV infection. IgM-class anti-p24-specific antibodies are noted from 16 to 122 days after seroconversion. IgG-class anti-HIV-specific antibodies arise from 18 to 144 days after seroconversion[34,90]. Spontaneous secretion of immunoglobulins of various classes tends to correlate negatively with the percentage—but not the absolute number—of CD4+ T cells[130]. In late-stage HIV infection, serum concentration of immunoglobulins may decrease. HIV-infected individuals have a relative or absolute lack of the IgG_2 and IgG_4 subclasses[7]. The IgG_2 subclass appears to be significantly decreased in AIDS patients with pyogenic infections[149]. It is unclear if elevated IgG and IgM levels have prognostic significance in HIV infection.

Although elevated serum IgA values have been observed in AIDS patients[50,53,93,181], such values do not generally appear early in HIV infection. Elevated levels may reflect an immune response to HIV, to opportunistic pathogens, or to a loss of immunoregulatory control of IgA production. Large cohort studies such as the Vancouver Lymphadenopathy Study (VLAS) of homosexual men[177,178], the Toronto Sexual Contact Study[27,29], the Canadian National Hemophilia Immune Study[197], and the United States Air Force[53] have noted an association between elevated IgA levels and subsequent progression to disease.

4.3 Circulating Immune Complexes

The humoral response to HIV infection results in the production of high levels of CICs. Several cross-sectional studies have noted an increased prevalence of CICs in HIV-infected individuals[73,123,213]. Using an assay based on complement- component-specific C1q mAb coupled to a solid phase to capture CICs from sera, the VLA(S) study noted higher levels of CICs in men that progressed to AIDS[177,178]. On the other hand, the San Francisco Men's Health Study did not find that elevated CICs were an indication of disease progression[172].

5. Antibodies to HIV

HIV-specific antibodies are produced early in the course of infection. Low serum titres of neutralizing antibody are observed as part of the humoral response to HIV. The antibodies that are formed are directed against all the major gene products: envelope products gp120 and gp41; pol products p66, p51, and p33; and gag products p55, p24, p18, and p15.

Studies of antibody response in early infection using numerous antibody assay systems (e.g., ELISA), indirect IFAs, RIPA, and Western blotting have shown a clear difference in test sensitivities[42,43,158]. The earliest antibody response is directed against gp160 and p24[65]. Since the first diagnostic test for HIV was licensed by the FDA in the United States for screening of sera, attempts to identify specific antibody profiles associated with disease stages have been sought[144,159].

HIV-specific antibodies that have been studied include

- anti-p24,
- anti-gp120,
- anti-p17,
- anti-gp41, and
- anti-NEF.

5.1 Anti-p24

Progression to AIDS is often associated with a decline in serum anti-HIV antibody titres, particularly anti-p24 core protein antibodies[114,179]. Decline in anti-p24 antibodies precedes p24 antigenemia and correlates with a poor prognosis in HIV-infected individuals[15,16,55]. In a British four-year prospective study of HIV-infected individuals, those with no anti-p24 antibodies or declining levels of anti-p24 antibodies had the worst prognosis. Poor prognosis was apparent as early as 27 months before the diagnosis of AIDS[210]. The British study did not find any association between clinical outcome and the presence of anti-gp41 and anti-gp120 antibodies. In addition, no decrease over time was detected in the titres of these antibodies. Because neutralizing antibody titres were unrelated to anti-p24 antibody titres, it was surmized that the protective effect of high anti-p24 levels is not mediated through a neutralizing mechanism.

5.2 Anti-gp120

Antibody response to HIV in haemophiliacs has revealed a strong association between the absence of anti-gp120 antibodies and progression to clinical ARC, such that anti-gp120 antibody levels may be of possible prognostic value[24]. However, this association was evident only when antibodies were screened by Western blotting of proteins separated under reducing conditions (a procedure that is not commonly used for screening sera from AIDS patients).

Approximately 70 percent of HIV-infected pregnant women fail to transmit infection vertically to their offspring. The lack of vertical transmission is correlated with high levels of neutralizing antibody specific for gp120 epitopes not involved in CD4 binding[205].

5.3 Anti-p17

In a small Dutch study of homosexual men, anti-p17 reactivity was detected as early as 10 months before disease development, and preceded anti-p24 decline[114]. Because anti-p17 antibodies have some virus neutralizing activity, declining titres of anti-p17 may signify reduced control of HIV pathogenesis.

5.4 Anti-gp41

The HIV envelope protein gp41 has sequence homology with p15E, a peptide derived from a C-type retrovirus that has immunosuppressive properties. It has been postulated that the presence of antibodies to pHIVIS, a 17-mer peptide derived from gp41, could neutralize the immunosuppression mediated by this peptide and thus affect the outcome of HIV disease and development of AIDS[25]. Two studies on homosexual men failed to find any association between antibodies to pHIVIS and HIV disease progression[23,113].

Antibodies to gp41 may be important in preventing vertical transmission of HIV infection to neonates[205].

5.5 Anti-NEF

Accessory gene products of HIV such as the protein NEF are also antigenic. NEF may play a role in regulating the expression of viral structural proteins. Antibody responses to NEF were present in 80 percent of individuals enrolled in a longitudinal study of a cohort of 194 asymptomatic HIV-infected individuals and 72 seroconverts. Anti-NEF antibodies were among the earliest antibody responses to HIV. In two of the seroconverts, antibodies of this specificity were detected together with anti-gag antibodies before seroconversion. Absent or transient responses to NEF were associated with absence or disappearance of anti-p24 core protein antibodies, reappearance of HIV core antigen, and decline in the number of CD4+ T cells[165,166]. The association of low or absent levels of anti-NEF antibody with poor prognostic marker profiles suggested a correlation between anti-NEF-specific antibody responses and disease progression. Closer examination revealed that this association was not significant.

Anti-NEF antibodies have been detected in groups that have no risk of HIV infection[160]. A sensitive liquid phase radio-immunoassay was used to examine serial serum samples from 12 individuals following seroconversion and 32 HIV-infected haemophiliacs. Anti-NEF antibodies were undetectable independent of the appearance of anti-gag, anti-pol, or anti-env-specific antibodies[10].

6. Other Antibodies

Evidence now exists that the HIV virus induces a wide range of autoimmune phenomena[133]. These phenomena include the appearance

- of lupus anticoagulant and anticardiolipin antibodies,
- anti-leukocyte antibodies,
- anti-soluble CD4, and
- anti-soluble CD8.

6.1 Lupus Anticoagulant and Anticardiolipin Antibodies

Elevated levels of lupus anticoagulant and anticardiolipin antibodies have been observed in HIV-infected individuals in high-risk groups[14,148]. Although these antibodies were originally thought to be early autoantibodies, they most likely represent an anti-phospholipid-specific response to viral infection in general, and do not have any prognostic value or significance[30,31,83].

6.2 Anti-leukocyte Antibodies

Anti-leukocyte antibodies are found in some HIV-infected individuals and have no prognostic significance[95,142,145,189]. Anti-platelet antibodies were identified in HIV-infected homosexual men[132,188]. Immune-mediated thrombocytopenic purpura was recognized early in the AIDS epidemic and was one of the diagnostic criteria for ARC[89,132,151]. Where classic autoimmune thrombocytopenic purpura involves anti-platelet IgG binding platelets, the thrombocytopenic purpura seen in AIDS and ARC patients is usually caused by non-specific deposition of complement components C3 and C4, and immune complexes onto platelets.

Immune neutropenia may, to a certain degree, be attributable to a similar mechanism[209].

6.3 Anti-Soluble CD4 and Anti-Soluble CD8

The main mechanism by which HIV induces immunodeficiency is the selective loss and functional impairment of CD4+ T cells. Indirect mechanisms of cellular destruction such as antibody-dependent cellular cytotoxicity may play a role in this process. For this reason, antibodies to CD4 have been sought at various stages of HIV infection using anti-recombinant sCD4 assays.

Antibodies to sCD4 have been found in only 5 to 12 percent of serum samples taken from HIV-infected individuals[193,212]. In one study of 253 seropositive individuals, the timing and appearance of anti-sCD4 had no effect on CD4+ T cell numbers or on disease progression[193].

The release of CD8 molecules from human T cells (i.e., soluble CD8 [sCD8]) can be measured by ELISA[64]. Levels of circulating sCD8 have been examined at all stages of HIV infection[146]. Irrespective of their symptoms, most patients had levels of sCD8 that were within 95 percent of that observed in uninfected controls. AIDS patients and asymptomatic HIV-infected individuals could not be separated based on levels of sCD8, suggesting limited usefulness for sCD8 measurement as an early surrogate marker for clinical AIDS development[120].

7. Other Serologic Markers

Other serologic markers for HIV infection include

- tumor necrosis factor (TNF-α),
- acid-labile human leukocyte interferon (IFN-α), and
- 2-5A synthetase.

7.1 Tumor Necrosis Factor (TNF-α)

TNF-α functions as part of a complex network of cytokines involved in the regulation of the immune system[9]. TNF-α is produced by macrophages and monocytes in response to naturally occurring infections. TNF-α generally upregulates macrophage and cytotoxic T cell potential[128,183]. Elevated levels of TNF-α have been found in the sera of AIDS patients, but not in early stages of HIV infection[105,129,161,162,163,171,214].

TNF-α may be responsible for myelin damage that occurs in HIV-associated encephalopathy[129] and may serve as a surrogate marker for monitoring the neurologic manifestations of HIV infection. The role of TNF-α in the pathogenesis of HIV infection is unclear. New immunoassays are currently being used to longitudinally screen collected sera from cohort studies, which could provide insight into the potential usefulness of TNF-α as a marker.

7.2 Acid-labile Human Leukocyte Interferon and 2-5A Synthetase (IFN-a)

Acid-labile human leukocyte interferon (IFN-α) and levels of 2-5A synthetase, an interferon-induced enzyme, are elevated in homosexual men infected with HIV[28,37]. In the Toronto Sexual Contact Study, acid labile IFN-α and 2-5A synthetase levels correlated with cervical lymphadenopathy and progression to AIDS[115]. IFN-α present in the sera of AIDS patients can induce the expression of TNF-α receptors and peripheral blood monocytes from HIV-infected patients; this induction is enhanced in later stages of disease. This activation of the TNF system may contribute to some of the physiological disturbances observed in AIDS patients[115].

8. Antigen Markers

8.1 p24 Antigen

The HIV-encoded gag gene product p24 is one of the first virally encoded molecules that can be detected in the circulation of infected individuals. This antigen is present transiently following initial infection with HIV; it reappears late in the disease[33,169]. Few patients have persistent p24 antigenemia. Although p24 antigenemia may be a poor prognostic sign, many patients do not develop AIDS for several years following detection of circulating p24[85,125,136].

Long-term AZT therapy may prolong survival and decrease the frequency of opportunistic infections, particularly pneumonia[51,143,174]. AZT treatment reduces circulating levels of p24 antigen, indicating that measurement of this marker may be useful in evaluating new anti-retroviral drugs[145,185]. However, one study reported that improvement in the number of CD4+ T cells and p24 antigenemia following AZT treatment did not predict the outcome in AIDS patients who previously had pneumonia[186].

Numerous epidemiologic studies have shown a clear correlation between falling CD4+ T cell numbers and the development of AIDS. Decline in CD4+ numbers appears to reflect an increase in HIV replication by correlation with increased serum concentrations of p24 antigen[40]. In a study of predictive markers of AIDS in haemophiliacs, p24 antigenemia and low CD4+ T cell counts were both very strong independent predictors of disease progression. Among seropositive haemophiliacs, from 17 to 18 percent had detectable serum p24 antigen[47,118,119]. The two-year actuarial incidence of AIDS was as follows:

- 24 percent after detection of p24 antigen,
- 16 percent after the loss of anti-p24 antibody,
- 20 percent after the loss of anti-gp120 antibody, and
- 31 percent after a decline in CD4+ T cell count to less than 200/mm^3.

The two-year AIDS incidence rose dramatically to 67 percent for those patients who were both positive for p24 antigen and whose CD4+ T cell count fell below 200/mm^3.

Although p24 antigen alone was highly specific for AIDS, sensitivity of detection was low. When p24 antigen was coupled with a low CD4+ T cell count, the specificity increased to almost 100 percent, but sensitivity fell to as low as 25 percent. Results obtained from this American study of patients with haemophilia was in agreement with other published studies of haemophiliacs or homosexual men in noting that p24 antigenemia and CD4+ T cell numbers were independently predictive of progression to AIDS[39,40,135,170].

In addition, the study noted that patients with p24 antigenemia and a very low CD4+ T cell number

had a high risk of developing AIDS (approximately 50 percent within one year and 67 percent within two years)[47]. This result is in contrast to a French study of haemophiliacs, where p24 antigenemia had predictive value but CD4+ T cell counts[2] did not.

In a large, multicentre prospective cohort study involving 1,219 subjects with haemophilia and related disorders, the eight-year mean cumulative rates for progression to AIDS were:

- 13 percent for ages 1 to 17,
- 28 percent for ages 18 to 34, and
- 44 percent for ages 35 to 70.

The presence of elevated serum interferon, elevated serum p24 antigen, low or absent anti-p24 antibodies, or low or absent anti-gp120 antibodies all had predictive value for the development of AIDS[71,150]. Adults older than 35 years had a higher incidence of low CD4 counts than younger subjects, whereas adolescents with low anti-p24 antibody levels had the lowest incidence of AIDS[71,153].

9. Cell Surface T Cell Activation Markers

The relationship between four serologic activation markers (sIL-2R, serum ß$_2$-M, serum neopterin, and sCD8) and T cell subsets were measured in a study involving 64 HIV-seropositive individuals and 61 seronegative controls[156]. Significant correlations were found in the HIV-seropositive group in pair comparisons of each of the serologic markers. CD4+ T cell numbers were negatively correlated with all four serological markers, but no correlation was detected between CD8+ T cell levels and any of the surrogate markers.

Significant correlations were discerned among various cell surface activation markers and serologic activation markers. The proportion of CD8+ CD45RA+ T cells was negatively correlated with neopterin and ß$_2$-M levels; the proportion of CD8+ HLA-DR+ cells correlated positively with ß$_2$-M and sIL-2R levels, while the proportion of CD8+ CD38+ T cells correlated positively with the four serologic activation markers[156]. A shift from naïve (CD45RA+CD45RO-) to memory (CD45RA+CD45RO-) T cell phenotype was seen in the CD8+ subset of HIV-infected individuals suggesting a disappearance of naïve resting CD8+ cells and an increase in the number of committed previously activated cells in this subset[157]. The expression of T cell-associated markers Leu-2 and Leu-7 were detected in seropositive haemophiliacs[195,196].

10. CD4+ T Cells

10.1 Technology

Technological advances of the early 1980s and the development of commercially available mAbs directed against specific lymphocyte cell surface antigens have led to a better understanding of immunodeficiency and lymphoproliferative disorders—diseases known to be associated with abnormal lymphocyte populations. The diagnosis and management of many of these disorders depends on serial monitoring of cell populations.

Sensitivity, speed, and precise quantitative capacity have made flow cytometry the method of choice for immunophenotyping. The transfer of this technology from the research laboratory to the clinical setting is, to a large measure, a result of:

- enhanced instrumentation,
- ease of machine operation,
- simplified calibration methods, and
- ongoing development of mAbs and fluorochromes.

The enumeration and analysis of cell populations have improved with the introduction of fast, powerful, affordable, and reliable microcomputers.

10.2 Protocol and Standardization

As the clinical applications of flow cytometry technology grow, so does the need for protocol and control standardization. Immunophenotyping is a sensitive assay; it is prone to errors resulting from improper sample collection, transportation, and preparation. Specimens must be processed quickly using appropriate safety precautions. Positive and negative controls are an essential part of the quality-control process and instrument function must be assessed daily[52,66,70,97,167].

Diurnal variation, stress, exercise, acute infections, and concomittant drug therapy can influence lymphocyte subpopulations. Fluctuations in lymphocyte subpopulations owing to these factors can be minimized by standardizing techniques for the drawing of blood, as well as by ensuring little procedural variation in the laboratory.

10.2.1 Defining T Cells

Common practice is now to use two-colour combinations to define CD4+ or CD8+ T cells as true subsets of CD3+ T cells. Furthermore, it has been suggested that a `lymphosum' be carried

out where the total of T, B, and natural killer cells should approach 100 percent[109]. A second sum of CD4+ and CD8+ T cells should equal the total number of CD3+ T cells ± 10 percent.

10.2.2 Determining the Level of Immunodysfunction

Because HIV targets and destroys CD4+ T cells, the most useful assay in determining the level of immunodysfunction in HIV infection is the phenotypic analysis of a patient's lymphocytes[140]. All T cells have distinctive cell surface glycoproteins that are important for their recognition. They express CD3, a glycoprotein previously known as T3 or OKT3, on their surface. These CD3+ cells can be subdivided into CD4-bearing and CD8-bearing cells. The CD4+ T cells usually have a helper function, while the CD8+ T cells usually function as cytotoxic or suppressor cells[164].

Early in the AIDS epidemic, before the identification of HIV as the pathogenic agent, persons thought to be at risk for AIDS were screened by determining the `helper/suppressor ratio,' or CD4+/CD8+ T cell ratio. Low values (below 1.0) in high-risk individuals raised suspicion of immunodeficiency. The identification of HIV as the causative agent of AIDS, and the subsequent demonstration that CD4 can bind the envelope of HIV (gp120), confirmed the importance of CD4+ T cells as targets of this virus[124]. The data accumulated from most large cohort studies made it clear that the severity of HIV-induced disease was associated with progressive depletion of both the percentage and absolute numbers of CD4+ T cells and that these cellular markers were powerful and independent predictors of progression to AIDS[49,135].

CD4+ T Cell and Total Cell Numbers

Percentage of CD4+ T cells, the absolute number of CD4+ T cells, and the CD4+/CD8+ T cell ratio all have excellent prognostic value for development of clinical AIDS and are highly correlated to each other[13,134,139,187,190,191]. Based on these findings, the percentage of CD4+ T cells has been recommended as the preferred prognostic marker, because this measurement has a lower variability than that for the absolute number of CD4+ T cells. Measurement of the percentage of CD4+ T cells also avoids the cost of a complete blood cell count.

Nevertheless, in most cases of HIV infection, it is recommended that total leukocyte and platelet numbers, as well as hemoglobin and CD4+ T cell counts, be monitored. The absolute number of CD4+ T cells has already become so widely used by clinicians and patients that it would be hard to discourage its use in favour of measuring CD4+ T cell percentages only. Most clinical trials include specific absolute CD4+ T cell numbers as entry requirements and as measures of outcome[4].

Predicting Progressive Immunodeficiency

The level of CD4+ T cells has strong predictive value as a marker of severe progressive immunodeficiency[103,108,139]. *Pneumocystis carinii* pneumonia, CMV pneumonia, and pulmonary infections with *C. neoformans* or *M. avium intracellulaire* rarely occur in HIV-infected patients with CD4+ T cell numbers greater than 250/mm^3 or a CD4+ T cell percentage of between 20 and 25[122,154]. This information can be used to formulate decisions on prophylactic treatment of individuals at high risk for *Pneumocystis carinii* pneumonia.

Uninfected individuals tend to have a baseline level of CD4+ T cells that is maintained over time, with minor fluctuations. As early as six months following seroconversion, infected individuals experience a decline in CD4+ T cell numbers[19,178]. Within two years, baseline levels of CD4+ T cells decrease by 30 to 50 percent[109]. Many asymptomatic HIV-infected persons maintain stable levels of approximately 600/mm^3 for many years[109]. However, some cohort studies have demonstrated slow, yet steady, declines in CD4+ T cell numbers even during the clinically stable latent period[113]. Healthy, asymptomatic individuals without p24 antigenemia and mean CD4+ T cell counts of approximately 520/mm^3 can exhibit short-term declines of 5 to 6 CD4+ T cells per cubic millimetre per month[198]. Rapid decline in CD4+ T cell numbers is a poor prognostic sign and may herald the development of opportunistic infections[139]. Following the diagnosis of AIDS, CD4+ T cell counts remain very low and can decline to undetectable levels before death.

In a British study of haemophiliacs, patients with CD4+ T cell counts of 200/mm^3 had a 5 percent cumulative risk of developing AIDS. This probability dramatically increased to 50 percent when counts fell to 50/mm^3, and subsequently increased to 81 percent when they dropped to 10/mm^3 [119,152].

It was initially hypothesized that certain functional subsets of CD4+ T cells, as defined by cell surface Leu 8, 2H4, or 4B4 expression, would provide additional markers for disease progression[36]. 2H4 defines the suppressor inducer subset of CD4+ T cells while 4B4 defines the helper inducer subset of CD4+ human T cells[131]. It has subsequently been demonstrated that all CD4+ T cell subsets decline with time and no further insight is gained by subdividing CD4+ T cells[68,69,207]. However, selective activation of subsets of CD8+ T cells—in particular HLA-DR+, CD38+, and Leu-8-CD8+ lymphocytes—were associated with a decline in CD4+ T cell levels and progression to AIDS[68].

Effects of AZT

Clinically beneficial effects of AZT have been demonstrated in patients with AIDS and ARC, as well as in asymptomatic individuals with CD4+ T cell counts of less than 500/mm^3 [199,200,202,206]. Based on this information, large segments of the HIV-infected population are currently receiving treatment with this drug. It is commonly believed that the effectiveness of AZT in treating HIV-

induced disease may be the result of an increase in CD4+ T cell counts or a slowing of the reduction in CD4+ T cell numbers. Modest increases of approximately 50 cells per cubic millimetre are detected in asymptomatic individuals treated with AZT if their baseline CD4+ T cell counts are less than 500/mm^3 [206]. For those with CD4+ T cell counts greater than 500/mm^3, no such increase is observed and an overall decline of 8.6 cells per cubic millimetre per month has been observed over a three-year AZT treatment period[200]. Haemophiliacs treated with AZT have also exhibited declines in their CD4 counts after treatment has been initiated[91].

One study examined the early effects of AZT therapy on five viral and immunologic markers of HIV activity in 90 patients with AIDS or ARC. These patients were followed for two years or until their deaths[88]. Changes in CD4+ T cell and lymphocyte counts and serum ß$_2$-M levels after 8 to 12 weeks of therapy could be used as surrogate end points for clinical outcomes in clinical trials. In 33 patients with a better pretreatment prognosis, the 24-month survival rate was 88 percent if they had a good response on these two surrogate markers early in treatment. Those with a poor response on either marker had a less than 50 percent chance for survival.

10.3 Drug Effects

Rapid and accurate identification of drug effects in patients being treated for HIV infection is desirable. It is preferable to reach conclusions about the possible benefit of drugs for HIV treatment without having to wait for survival differences among patients. Availability of this information would permit accelerated screening for effective drugs and allow for earlier regulatory approval. More studies are in progress to ascertain whether a change in surrogate marker values over time correlates with long-term survival. This information is urgently needed. Analyses of large placebo-controlled trials will determine the usefulness of markers as clinically meaningful end points.

11. Discussion and Recommendations

The usefulness of surrogate markers for clinical disease development in various areas of research is well established. Blood pressure measurements and cholesterol levels have proven clinical value in determining the long-term outcome with respect to cardiovascular disease. The use of surrogate markers as end points in HIV clinical trials has the potential of reducing both the cohort size required to conduct such studies, and the duration of each trial[201]. The ability to carry out small and quick studies has appeal not only to patients but also to clinical researchers and pharmaceutical firms. Industry would benefit by having products evaluated rapidly and thus at less cost. Government drug regulatory agencies may show an interest in such trials, as they would allow for the rapid licensing of drugs.

Why then, have surrogate markers not been used as end points in HIV clinical trials? The answer is that we lack sufficient knowledge about the illness. Because clinical changes are apparent only years after infection, the study of these markers and an attempt to understand their usefulness in the natural history of this disease has taken years to accomplish. Furthermore, treatments for HIV have only recently begun, resulting in a limited understanding of the effect of therapy on these markers.

To be of clinical use, surrogate markers must fulfill certain criteria:

- They must have a clear, logical, and pathophysiologic association with the disease process.
- They must have a clear role in the natural history of HIV-induced illness.
- They must be detectable in the majority of infected individuals.
- They must change, measurably, with clinical status both in the progression and remission of disease.
- They must change quantifiably following successful therapeutic intervention, or not change following failure of therapy.

Because these criteria have not been met for any single marker, it is not surprising that surrogate markers are not, as yet, accepted primary end points in clinical trials. Measurement of surrogate markers has only recently been proposed for use in phase II (but not phase III) studies[21,127].

Appraisal of surrogate markers of HIV-induced disease should have a place in the management and treatment of early and mid-stage infection, as they may provide clues regarding the state of the immune system in individuals with asymptomatic HIV infection. Surrogate marker quantitation may provide valuable insights when used in conjunction with clinical assessments, such as quality-of-life scores, functional status, and weight, which are not specific for HIV-induced disease. Measurement of surrogate markers for development of clinical AIDS may also indicate the effect of drug (or other) therapy within weeks of initiation of treatment[38,88,106,180].

In studies of the natural history of HIV infections, levels of the most commonly surveyed surrogate markers correlate with survival time. It remains to be seen if treatments that return surrogate marker levels to those seen in uninfected individuals result in improved survival. Indeed, administration of AZT results in improved survival but not in a sustained maintenance of high CD4+ T cell counts[91]. Recently, a correlation was demonstrated between the effect of AZT on surrogate marker values early in treatment and long-term survival[88]. Such studies must be validated in larger placebo-controlled cohorts before surrogate marker measurement can be accepted in the place of clinical end points.

When the prognostic value of three cellular and five serologic surrogate markers in HIV infection were evaluated, CD4+ T cell levels (expressed as an absolute number, or a percentage of lymphocytes, or a ratio of CD4+ to CD8+ T cells) were the best single predictor of progression to AIDS. A step-wise multivariate analysis indicated that the best predictors in descending order were:

- levels of CD4+ T cells;
- serum level of neopterin or $ß_2$-M;
- level of IgA;
- sIL-2R; and
- p24 antigen.

Fahey and co-workers concluded that the level of CD4+ T cells in combination with a serum level of either neopterin or $ß_2$-M were the most powerful predictors of progression to AIDS[49].

CD4+ T cell counts, $ß_2$-M, neopterin, and p24 antigen are now measured in clinics and used singly or in combination to follow patients and make therapeutic decisions such as when to begin anti-retroviral therapy or prophylaxis for *Pneumocystis carinii* pneumonia[5,6,47,48,49,51,88,100,101,102,143].

If these markers are to be used as primary end points in clinical studies, issues such as standardization of tests must be addressed. Consensus must be sought for methods, reagents, and equipment. Strict quality-control guidelines should be encouraged. Laboratory normal ranges must be defined for each laboratory, and concomitant testing of both positive and negative controls should occur in concert with patient testing. Laboratory proficiency testing and accreditation should be required particularly for multicentre trials in particular. At each testing

site, sequential sampling is important, as single measurements have little meaning. Although trends may have value, a consensus must be reached for definite cut-offs in clinical studies. These laboratory end points must be confirmed by repeat short-term sampling. Each marker must be validated in all high-risk groups with the appropriate adjustments. The stability of serologic markers in frozen samples must be shown, and then serologic assays should test sequential series of patient samples through batch processing. For example, an absolute CD4+ T cell count of $200/mm^3$ or 20 percent CD4+ T cells could be an end point indicating failure to affect asymptomatic disease. This end point must be confirmed by at least two repeat counts in this range within a period of two to four weeks.

In conclusion, direct assessment of immune deficiency status through measurement of CD4+ T cell percentage or absolute number will continue to be the preferred surrogate marker of clinical AIDS development in the near future, owing to wide availability of the test, its affordability, and our current level of understanding. It is unlikely that indirect measures of disease activity such as $ß_2$-M or neopterin will be acceptable for use as end points in clinical trials in the near future. Direct measures of virus activity such as viremia, although more acceptable, are not generally available and are very costly.

12. Glossary

$ß_2$-M	$Beta_2$-microglobulin
2H4	Marker for suppressor inducer CD4 subset
4B4	Marker for helper inducer CD4 subset
ARC	AIDS-related complex
AZT	Zidovudine
C1q mAb	Monoclonal antibody to the CIg component of complement
CD4	T helper subset marker
CD8	T cytotoxic/suppressor subset marker
CIC	circulating immune complex
CMV	cytomegalovirus
CSF	cerebrospinal fluid
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunoassay
FDA	Food and Drug Administration
GTP	guanosine triphosphate
HIV	Human Immunodeficiency Virus
HPLC	high-performance liquid chromatography
HTLV-1	Human T-lymphotropic Virus type 1
IFA	immunofluorescence assays

IFF-	gamma-interferon
IFN-α	alpha-interferon
Ig (IgG, IgM, IgA)	Immunoglobulin
IL-2	interleukin-2 (T cell growth factor)
IL-2R	interleukin-2 receptor
IL-2Rα	interleukin-2 receptor alpha chain (Tac peptide)
IL-2Rß	interleukin-2 receptor Beta chain
IL-6	interleukin-6 (a B cell stimulatory factor)
Leu 8	T cell subset marker
mAbs	monoclonal antibodies
MHC	major histocompatibility complex
NEF	negative factor
pHIVIS	17-mer peptide of HIV gp41
RIPA	radio-immunoprecipitation
sCD4	soluble CD4
sCD8	soluble CD8
sIL-2R	soluble interleukin-2 receptor
TNFα	tumor necrosis factor alpha
VLAS	Vancouver Lymphadenopathy Study

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