Infection

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Prognostic Parameters
for Therapy Study of HIV Infections

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Lymphocyte Subsets as Surrogate Markers in Antiretroviral Therapy

Summary: Efficacy of antiretroviral treatment is evaluated usually according to reduction of serious events (e.g. opportunistic infections while on therapy) and improvement of survival time. In stages of asymptomatic disease treatment trials have to cover very long time periods to fulfil these requirements. In asymptomatic stages, when viremia is commonly absent, monitoring the host's immune response is an indirect means of measuring antiviral efficacy. $CD4+$ lymphocyte counts are generally accepted as surrogate in all major trials. The subsets of the $CD8+$ compartment reflect early and late activation and cytotoxic immune response. $CD38+$, CD57, $CD8+HLA/DR+$ subsets reflect the host's vigorous cellular immune response even in early stages. These subsets are candidate surrogate markers in early a n d late stages of HIV infection. On the other hand, $CD3+CD4- CD8-$, CD19/20 (B lymphocytes) and $CD16+$ (natural killer cells) do not exhibit any properties of candidate surrogate markers. Established and experimental cellular surrogate markers are discussed including own data and a review of the literature.

Zusammenfassung: *Lymphozytensubpopulationen als Surrogatmarker bei antiretroviraler Therapie.* Die Beurteilung der Effektivität einer antiretroviralen Therapie geschieht überwiegend anhand der Krankheitsprogredienz und der Überlebenszeit. Es besteht Interesse an Surrogatmarkern, die eine Aussage zu Wirksamkeit der Therapie ermöglichen, bevor es zu einer Progredienz kommt und solange ein asymptomatisches Stadium vorliegt. Gerade während asymptomatischer Stadien bieten sich Parameter, welche die Immunantwort auf die HIV-Infektion widerspiegeln, hierfür an, da eine Virämie meist noch nicht vorliegt. Die einzigen derzeit etablierten Surrogatmarker unter den Lymphozyten-Subsets sind $CD4+$ und $CD8+$ Lymphozyten. Anhand eigener Ergebnisse und Diskussion der Literatur wird auf die Möglichkeit neuer Subset-Untersuchungen eingegangen: Die Aufgliederung der $CD8+$ Zellen mit Markern für Aktivierung und Zytotoxizität (HLA/DR, CD38, CD57) ermöglicht eine stadienabhängige Beurteilung der Beeinflussung von zytotoxischer Immunantwort als Auswirkung der HIV-Immunpathogenese. Andere Subsets, wie beispielsweise B-Lymphozyten, CD3+4-8-Lymphozyten und Killerzellen eignen sich theoretisch weniger gut als Surrogatmarker.
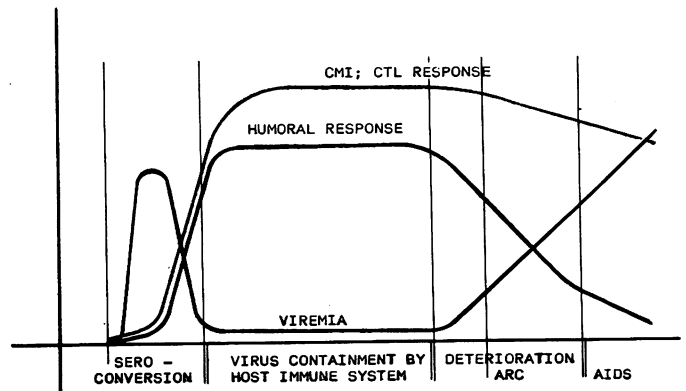
Figure 1: Model of viremia and host immune response in the course of HIV infection. The asymptomatic period is characterized by efficaceous virus containment due to humoral and cell mediated immunity. In this stage monitoring of the host's immune response serves as candidate surrogate for antiviral efficacy (rather than parameters of viremia).

CMI = cell mediated immune response; CTL = cytotoxic lymphocyte; ARC = AIDS related complex.

clue to diagnosis (*Pneumocystis carinii* pneumonia, candidiasis, Kaposi's sarcoma). A profound change in the relation of suppressor and helper T lymphocytes resulting in an inversion of the ratio of $CD4+$ and $CD8+$ cells was the immunological "surrogate" for the syndrome when no clinical signs were apparent in patients at risk [1].

In addition to this quantitative disturbance, a decreased lymphocyte proliferation was observed upon stimulation with mitogens and antigens *in vitro*.

After almost one decade the natural history is known quite well in many facets. The corresponding immunopathogenesis is characterized in many aspects, including the sequential changes of cellular immunity in the course of HIV infection. Efficacy of antiviral treatment is evaluated usually according to reduction of serious events (e.g. opportunistic infections while on therapy) and prolongation of survival. In stages of asymptomatic disease, treatment trials have to cover very long time periods to fulfil these requirements. Reliable surrogates indicating antiretroviral efficacy therefore could contribute to a better feasibility of clinical trials. Theoretically, virological and immunological parameters are not equally useful as surrogate markers in every stage, as demonstrated in **Figure 1**: antigen detection is more likely in symptomatic disease as compared with asymptomatic stages.

Introduction

When AIDS was first discovered clinical diagnoses of infections and neoplasms due to immunodeficiency were the

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Table 1: Quantitative changes of lymphocyte subsets in the course of HIV infection.

Lymphocyte subset		Kinetics in HIV infection
CD4+	*	Depletion
CD4+CDw29+	+	Earlier depletion
CD3+CD4−CD8−	−	Minor expansion
CD8+HLA/DR+	+	Early expansion
CD8+CD38+	+	Late expansion
CD8+CD57+	+	Expansion
CD3+CD8+CD25+		?
HLA/DR+	−	
CD16+ (NK)	−	No or only slight quantitative change
CD19; CD20	−	No or only slight quantitative change

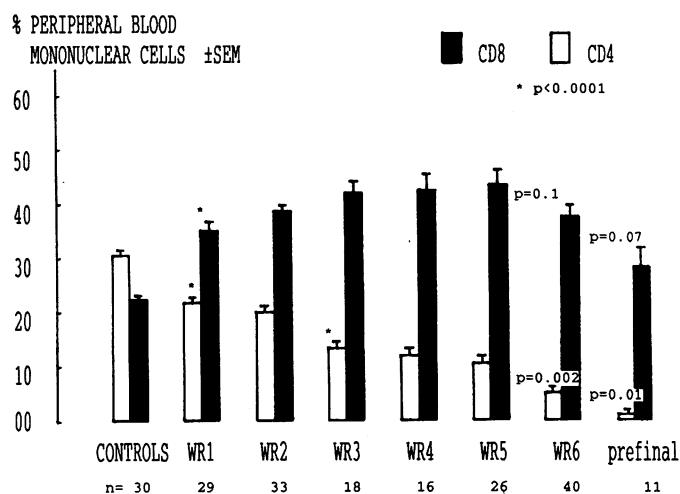Generally accepted (*) and candidate (+) surrogate markers; (−) = no properties of a surrogate.

Figure 2: Proportion of CD4+ and CD8+ lymphocytes in the course of HIV infection according to Walter Reed (WR) stages. In WR 1 significant differences as compared with controls could be detected.

SEM = standard error of the mean.

In earlier stages of HIV infection the monitoring of the host's immune response (humoral and cellular) is possibly a suitable way of monitoring the effects of viral burden and immunopathogenicity of HIV disease. The determination of quantitative and qualitative changes of cellular immunity as a possible surrogate marker of efficacy in antiretroviral therapy will be discussed in this paper.

Quantitative Kinetics of Lymphocyte Subsets in the Course of HIV Infection

An overview on increase and decrease of lymphocyte subsets during HIV infection is given in Table 1. Expansion of lymphocyte subsets mainly occurs in CD8+ lymphocytes and in the corresponding subsets of CD8+ cells and activated T lymphocytes. Most attention, however, is usually paid to the loss in percentage and absolute numbers of CD4+ lymphocytes.

CD4+ Lymphocytes

Helper lymphocytes (CD4+) constitute the center of cellular immune responses. Even humoral responses are facilitated or at least enhanced by CD4+ lymphocytes. The depletion of this subset of T lymphocytes results in a major immunodeficiency mainly of the cellular responses. Furthermore, the CD4+ cell with its CD4 molecule is the main target for HIV [2]. The severity of CD4+ cell depletion is taken as a parameter for the severity of immunodeficiency and for prognosis. This is why this number has been introduced in one of the major classification systems, the Walter Reed (WR) staging classification [3]: the number of 400/μl makes up the discrimination between stage WR 2 and WR 3.
CD4+ lymphocyte subset results of our patients in WR 1 are significantly lower than in controls (p<0.001; Figure 2), indicating an early reduction of this subset [4]. Lang and colleagues studied 39 seroconverters [5]. An early and

sustained reduction could be observed. This result was also confirmed by data published from the Multicenter AIDS Cohort Study. Within 12 to 18 months after seroconversion the reduction of CD4+ cells reaches approximately 60% of the baseline count prior to seroconversion [6]. The count then stays relatively stable during the subsequent asymptomatic years (Figure 1) [7]. The period before the onset of ARC and AIDS is characterized by a rapid CD4+ cell depletion [8].
The application of the CD4+ count as a surrogate marker for efficacy of antiretroviral treatment mainly depends on the stage studied. In the past most clinical trials were supplemented by determination of this parameter before and during therapy. Generally an expansion of the CD4+ subset is taken as a sign of efficacy [8, 9].
Zidovudine therapy, for example, results in a minor increase during the first months of treatment. The mean CD4+ count rose from 356 ±201/μl to 435 ±288/μl in long-term treatment of our patients (Figure 3).
The use of CD4+ counts as surrogate markers is an indirect method for rating the antiviral efficacy: if the CD4+ cell depletion is caused by increased viral burden, an inversion of this process should be taken as a sign of antiretroviral effects.
However, early treatment in stages of stable CD4+ count might cause difficulties in evaluation (Figure 4): in a period characterized by stable CD4+ counts an expansion seems less probable: in the trial of zidovudine in asymptomatic patients with CD4+ lymphocytes more than 200/μl an increase of helper cells could be shown statistically only in qualitative nonparametric testing but not in a quantitative comparison [9].
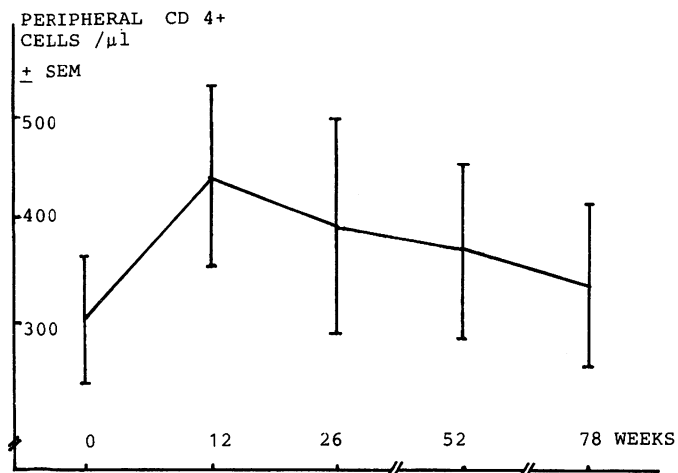
Figure 3: Expansion of CD4 + lymphocytes in patients on zido-vudine treatment.

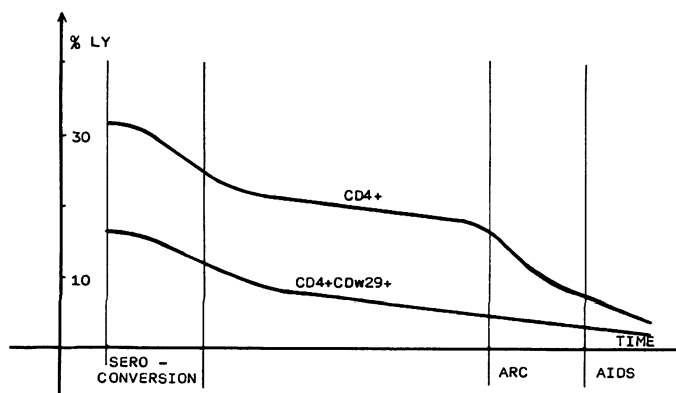n = 10; SEM = standard error of the mean.

Figure 4: Model of depletion of CD4 + lymphocytes in the course of HIV infection [4–7, 10]. The loss of CD4 + CDw29 (memory) cells accounts for a major part of the early CD4 + depletion [10].

Evidence is given in recent studies, that the CD4 + CDw29 + subset within the CD4 + lymphocytes is decreased at first (Figure 4) [10]. Therefore, this particular subset is a candidate surrogate marker in early treatment trials. Detailed knowledge about the kinetics of CD4 + CDw29 + lymphocytes during antiretroviral treatment could be the subject of any further trial in asymptomatic patients. Functionally the CD4 + CDw29 + cells show strong helper function for the induction of IgG response to recall antigens (memory function).

CD8 + Lymphocytes

The application of two and three color flow cytometry detects further subsets of the CD8 + compartment. Addi-
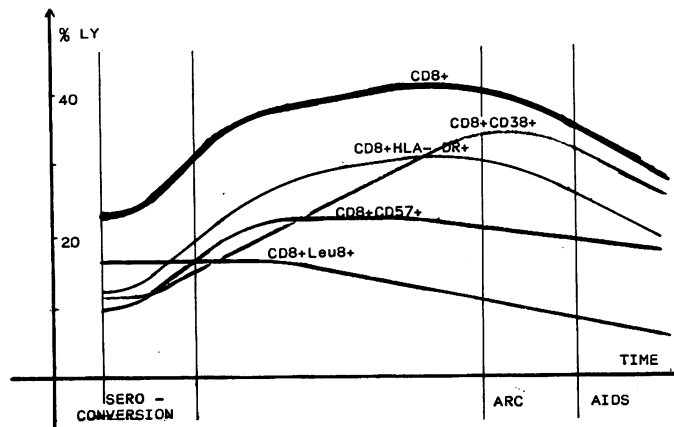

Figure 5: Model of the expansion of CD8 + lymphocytes in the course of HIV infection [4–6, 14]. CD8 + subsets bearing markers of activation and cytotoxicity expand in early (CD57 +, HLA/DR +), intermediate (CD5 +) and late (CD38 +) stages of HIV infection [4, 14].

tional monoclonal antibodies directed against surface molecules typical for a state of activation of cytotoxicity are Leu17 (CD38), Leu7 (CD57), Leu8, and HLA/DR.
CD38 occurs on activated T lymphocytes, thymocytes, natural killer (NK) cells and on some B lymphocytes. CD57 is a marker for cytotoxic T lymphocytes (in combination with CD8 and/or CD3). On the surface of T lymphocytes HLA/DR is a marker of cell activation [11].
Initially the inversion of the CD4/CD8 ratio was regarded as the hallmark of the acquired immunodeficiency and the major explanation was seen in the CD4 + depletion. However, as early as 1983 to 1985 some reports pointed out that not only the relative but also the absolute CD8 + counts were expanded [12, 13].
CD8 + counts of 1500 to 2500/μl are commonly found in HIV-infected patients. The (falsely) low determination of CD8 + cells after lymphocyte separation on a Ficoll gradient is corrected by a wide use of the lysed whole blood preparation (in which the obvious loss of CD8 + cells does not occur).
Up to 70% of circulating lymphocytes are CD8 + cells in some patients. The expansion of the CD8 + compartment is an event of early HIV infection. Again, a significant increase is found as early as WR 1 [4]. Monitoring the lymphocyte subsets in seroconverters reveals an increase already at seroconversion [5, 6] (Figure 5).
A further development of the CD8 + compartment shows a stage dependent sequential pattern [14–16, 4) (Figure 5). Thus the early expansion of CD8 + cells is caused by an increase of CD8 + Leu8– lymphocytes, CD8 + HLA/DR + and CD8 + CD58 + (Figure 5). The ratio of CD8 + Leu8 + to CD8 + Leu8– lymphocytes decreases. The CD8 + Leu8– subset parallels the lymphocyte depletion and does not participate in the CD8 + expansion [14]. CD8 + CD38 + lymphocytes increase with progression towards full-blown

AIDS. In the late stages ARC and AIDS most of the CD8+ cells also carry the CD38 molecule (Figure 5). Two colour flow cytometry using CD57 shows that CD8+CD57+ as well as CD8+CD57– cells expand in early HIV infection [15,16,4]. A similar expansion is seen in rejection of renal allografts [17]. Functionally CD8+CD38+ and CD8+CD57+ lymphocytes are involved in cytotoxicity against HIV-infected cellular targets [15]. In addition, antibody dependent cytotoxicity (ADCC) is triggered by antibodies to HIV antigens and may be conveyed by this cell type. Lymphocyte autoantibodies against CD4+ lymphocytes [18] contribute to cytolysis and helper cell depletion (even of noninfected lymphocytes).

In ten of our ARC patients treated with zidovudine (1000 mg/d) the average proportion of HLA/DR+ T lymphocytes dropped from 43.9% to 38.4% of peripheral lymphocytes after three months of therapy (p = 0.07, Mann-Whitney Test). However, this result has to be compared with a control group. While according to the natural history in ARC a steady or even increasing count of HLA/DR+ T lymphocytes is to be expected, this result shows a decrease, most probably as an effect of treatment (Figure 6).

A small subset within the CD8+ compartment characterized as CD8+CD3+CD25+HLA/DR+ has been shown to exert a suppressive action on HIV replication *in vitro* [19]. Less than 10% of all CD8+ cells are of this phenotype. *Walker* and colleagues presented data on a soluble factor in the supernatant of cloned CD8+CD3+CD25+- HLA/DR+ cells acting in a suppressive fashion.

Knowledge presented here on the kinetics and subsets of CD8+ lymphocytes refers to the natural history of HIV infection. Data on CD8+ counts as surrogate marker for antiretroviral therapy are exceptional while the ratio of CD4+ and CD8+ cells has often been monitored in clinical trials. The Laennec HIV Study Group reported on cyclosporine treatment of HIV infected patients: CD8+ lymphocytes decreased significantly while CD4+ cells of patients with a baseline count of 300 to 600/μl showed a lasting increase [20]. Both subsets returned to baseline levels after three to six months of treatment.

Our own data on ARC patients treated with zidovudine are shown in Figure 6: after one year of treatment the average CD8+ count rises. Further studies should include a differentiation of the CD8+ subsets: a disproportional decrease of CD8+CD38+ and CD8+CD57+ cells in combination with a normalizing Leu8+/Leu8– ratio could indicate an effect on the immunopathogenesis (in contrast to a progression according to natural history).

CD3+CD4–CD8– Lymphocytes

CD3+CD4–CD8– lymphocytes (double negative T lymphocytes) expand in the course of HIV infection in peripheral blood as well as in lymph nodes [21,22]. A very
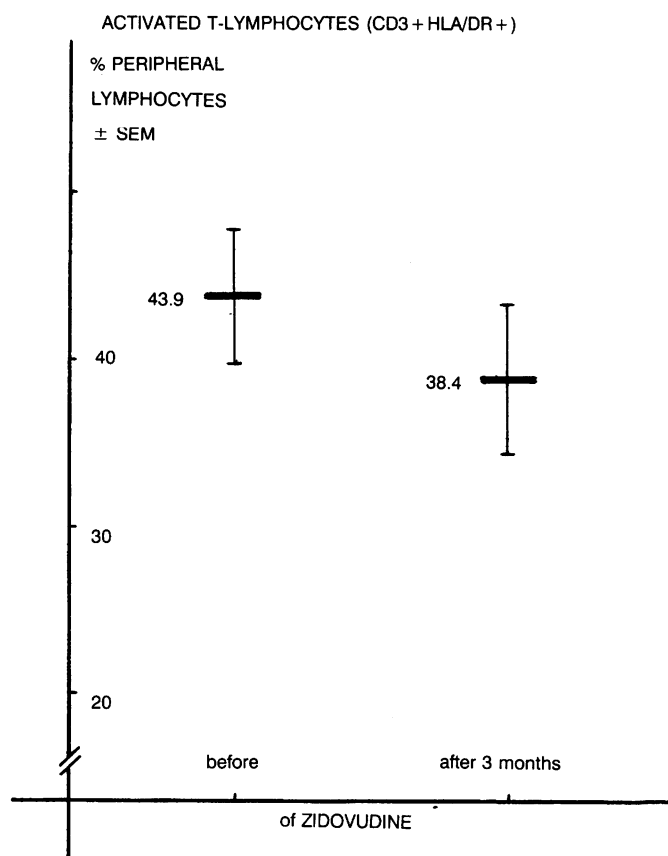

ACTIVATED T-LYMPHOCYTES (CD3+HLA/DR+)

Figure 6: Activated T lymphocytes (CD3+HLA/DR+) before and after 3 months of zidovudine therapy.

n = 10; SEM = standard error of the mean; p = 0.07.

small proportion of T lymphocytes usually bears this phenotype (<3%). *In vitro* CD3+CD4–CD8– cells develop to either CD4+ or CD8+ lymphocytes. The expansion of this subset represents the increased turnover of T lymphocytes [21]. While theoretically CD3+CD4–CD8– cells might serve as a surrogate marker, practical application is limited: the proportion of CD3+CD4–CD8– cells is a computed number. The difference of all CD3+ cells and the sum of CD4+ and CD8+ results in the number of CD3+CD4–CD8– cells [22]. Because of electronical gating in flow cytometry results are not reliably reproduced in single determinations.

Other Subsets

Lymphocyte subsets of non-T-cell origin like B lymphocytes (CD19+, CD20+) or natural killer (NK) cells (CD16+) do not vary quantitatively in the course of HIV infection. Thus, they cannot be regarded as candidate surrogate markers. The proportion of another type of lymphocytes, the "unstained cells" (morphology like lymphocytes but without binding of any monoclonal antibodies

against lymphocyte markers) rises towards final stages. Up to now, there is no experience as to whether this subset is useful as a surrogate in corresponding stages.

In Vitro Proliferation of Lymphocytes

The functional disturbance of the immune response caused by HIV infection mainly is known as diminished proliferation upon mitogen and antigen stimulation [12]. This phenomenon is not necessarily linked to T-cell or helper-cell depletion. *Teeuwsen* [23] and colleagues presented findings indicating reduced proliferation three months after seroconversion. *Clerici* et al. pointed out four different patterns of dysfunction independent of the WR stage or CD4 + count [22].

T-cell colony formation (T-CFC) of peripheral mononuclear cells is reduced in patients with HIV disease. *E. M. Levy* and colleagues observed the restoration of T-CFC in the course of zidovudine treatment of three months. This assay seems to be a sensitive surrogate of antiretroviral efficacy [24].

General Considerations

The use of lymphocyte subsets as surrogate markers is only possible when basic requirements are met: the method of lymphocyte preparation and differentiation should be reproducible and well established. A problem of multicenter studies is the diversity of methods (e.g. monoclonal antibodies from different clones and of different affinity) in participating immunological laboratories.

Absolute and relative counts of lymphocyte subsets may differ in reproducibility. Any discordance in the leukocyte differential count contributes to a multiplicative error in the computed absolute number. In a clinical trial absolute as well as relative counts should be monitored.

Whenever lymphocyte separation is necessary for a test (e.g. lymphocyte culture or stimulation) at least 20 ml of peripheral blood are required. The test therefore cannot be applied for a frequent follow-up because patients tend to be anemic anyway.

Acknowledgement

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