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The breakdown of the cytokine network subsequent to human immunodeficiency virus infection

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THE acquired immunodeficiency syndrome (AIDS) is a clinically multifaceted disease induced by infection with the human immunodeficiency virus (HIV). HIV infection results in a complex pattern of immunologic alterations that leads to the development of AIDS in the majority of HIV seropositive (HIV+) individuals. The reduction in CD4 T lymphocyte counts is the hallmark of HIV infection; nevertheless, long before the reduction in CD4 counts reaches critical levels, a series of profound and complex defects that impair the function of CD4 T lymphocytes can be detected. Thus, HIV infection is characterized by quantitative and qualitative defects affecting CD4 T lymphocytes. It was suggested recently that programmed cell death (PCD) is an important mechanism leading to CD4 depletion in HIV infection, and that susceptibility of peripheral lymphocytes to PCD is differentially regulated by diverse cytokines. Thus, type 1 cytokines would protect CD4 lymphocytes against PCD, whereas type 2 cytokines would not protect against, and could augment, PCD. We suggest that the qualitative alterations of the immune response provoke the CD4 depletion characteristic of HIV disease via type 2 cytokine-mediated augmentation of PCD, and are therefore ultimately responsible for the progression of HIV infection. Finally, we summarize recent data showing that three correlates of disease progression: emergence of HIV strains with syncitium-inducing ability (SI), type 1-to-type 2 cytokine shift, and CD4 depletion, are significantly associated, suggesting a complex interconnected virologic–immunologic pathogenesis of HIV infection.

Key words: Breakdown, Cytokine network, HIV.

Defective interleukin-2 production as the hallmark of qualitative lymphocyte defects in HIV infection

Early and complex T helper (Th) cells defects have been described in HIV infected individuals.[1–6] We have analysed the qualitative defects of HIV infection by examining *in vitro* antigen- and mitogen-stimulated interleukin (IL)-2 production by PBMC of HIV+ individuals. PBMC were stimulated *in vitro* with recall antigens (influenza virus, tetanus toxoid, or HIV peptides); HLA alloantigens (ALLO); or phytohaemagglutinin (PHA).[5] These stimuli were chosen because they activate different Th-antigen presenting cell (APC) pathways,[7] allowing for a more complete evaluation of the immune system. Thus, recall antigens are exclusively presented by self-APC to autologous CD4; HLA alloantigens can be presented by self-APC to autologous CD4 or they can be directly recognized by autologous CD4 and CD8 T lymphocytes on the surface of allogeneic APC; whereas PHA stimulation of T lymphocytes is only marginally dependent on processation and presentation by APC (see Table 1).[7]

The stimulation of PBMC with this panel of antigens allowed us to recognize that only a minority (≈40%) of HIV+ asymptomatic patients can respond by IL-2 production to recall, ALLO and PHA.[5] Thus, a complex pattern of defects in IL-2 production was observed in the majority of HIV+, asymptomatic individuals. Indeed, approximately 40% of these individuals showed a selective defect in IL-2 production in response to recall antigens, whereas ≈10% of them could only respond to PHA, or could not produce IL-2 in response to any stimulation (≈10%).[5] Because all these patients were in the same clinical stage (Walter–Reed stage 1), and

Table 1. Pathways of T helper cell activation

Antigen	T helper cell	Antigen presenting cell	IL-2 production
Recall antigens	CD4+	self APC	++
HLA alloantigens	CD4+	self APC	++
	CD4+	allo APC	+++
	CD8+	allo APC	++

PHA-stimulated IL-2 production is marginally dependent on processation and presentation by APC.

had comparable CD4 counts (>400 mmc) the observed functional defects were not secondary to a decrease in CD4 counts.[5] These experiments allowed us to conclude that: (1) defective ability to produce IL-2 can be detected even in the earlier, asymptomatic phases of HIV infection; and (2) these defects are not secondary to either a decrease in CD4 counts or a clinical progression in HIV infection. Similar analyses in vertically transmitted paediatric HIV infection showed that: (1) defective IL-2 production is observed also in the majority of HIV+ paediatric patients;[8] and (2) defective *in vitro* antigen- and mitogen-stimulated IL-2 production is associated in paediatric HIV disease with an augmented incidence of opportunistic and bacterial infections.[8] In the attempt to analyse whether the defective element is the T lymphocyte or the antigen presenting cell, we performed experiments in which cells of HIV-discordant monozygotic twins were matched. The results repeatedly indicated the absence of defects in the ability of HIV+ APC to process and present antigens.[9,10]

We subsequently verified whether defects in IL-2 production could be reverted by treatment with antiretroviral drugs. Thus, we measured *in vitro* stimulated T lymphocyte proliferation and IL-2 production in two cohorts of HIV+ adults and one cohort of paediatric patients, treated with zidovudine, sCD4–IgG or ddI respectively. We observed that all three compounds were capable of temporarily restoring IL-2 production independently of any variation in CD4 counts.[11–13] That this is not an aspecific effect of all antiretroviral drugs was shown by a fourth compound, which was not able to restore T lymphocyte proliferation or IL-2 production (M. Clerici *et al.*, unpublished observations). Additionally, the *in vitro* restoration of IL-2 production by antigen- or mitogen-stimulated peripheral blood mononuclear cells provoked by ddI was associated in paediatric HIV infection with a significant reduction of opportunistic infections during clinical follow-up.[12] Again, the positive changes in clinical parameters was associated with improved T lymphocyte function, but independent of variations in CD4 counts.

The type 1/type 2 hypothesis of HIV infection

In the late 1980s it was shown by different investigators that murine T helper cells can be functionally distinguished in two subsets which are differentially specialized in furnishing preferential help to cytotoxic T lymphocytes (CTL) or B lymphocytes.[14–17] These two sub-populations, named T helper 1 (TH1) and T helper 2 (TH2), respectively, were subsequently described in humans by Romagnani.[18,19] It was suggested recently that some phenotypic markers may allow us to differentiate TH1 and TH2 lymphocytes. Thus, it was reported that TH2 lymphocytes may express higher quantities of CD30,[20] as well as of a particular isoform of BB-7 (BB-7.2). A third group has observed that CD4+CD7+ T lymphocytes preferentially secrete TH1 cytokines, whereas CD4+CD7− T lymphocytes preferentially produce TH2 cytokines.[21] Nevertheless, no marker has yet been identified that exclusively clusters on TH1 or TH2, and the definition of TH1 and TH2 lymphocytes is still essentially based on the different cytokines that are produced by these cells. Thus, TH1 responses are characterized by secretion of interferon (IFN)-γ and IL-2 and subsequent promotion of cell mediated immunity (CMI), whereas TH2 responses are characterized by secretion of IL-4 and IL-5, with subsequent activation of humoral immunity and generation of antibodies. Interestingly, TH1 and TH2 are cross-regulating as IFN-γ suppresses the activation of TH2 lymphocytes, whereas IL-4 suppresses the production of TH1 cytokines.

IL-2 is a prototypical product of TH1 lymphocytes, and IL-2 production can be down-regulated by TH2 cytokines (classically IL-4). Thus, we began exploring the possibility that the defective IL-2 production observed in HIV+ asymptomatic infection could be accompanied by augmented IL-4 production. We decided to analyse cytokine production by whole PBMC and not by CD4+ T cell clones because we believe cytokine production by whole PBMC to be a more important approximation to the *in vivo* situation.[22,23] In fact, even if T cell clones are an important experimental tool, we were concerned about the artefacts present in the cloning methodology. Our concerns about clones can be summarised as follows: (1) Th cell cloning results in the loss (during the cloning process) of important, non-T accessory cells that produce cytokines with immunoregulatory properties, (2) cloning may

Table 2. TH1 and TH2 cytokines are clonally defined; type 1 and type 2 cytokines are functionally defined

TH1 cytokines	TH2 cytokines
Interferon γ	Interleukin-4
Interleukin-2	Interleukin-5
Type 1 cytokines	**Type 2 cytokines**
Interferon γ	Interleukin-4
Interleukin-2	Interleukin-5
Interleukin-12	Interleukin-6
Interleukin-15 (?)	Interleukin-10
	Interleukin-13 (?)

select for populations of T cells, as the techniques involve the selection and expansion of cells in relatively high concentrations of IL-2; and (3) the investigation of PBMC tests for the effects of cytokines produced by multiple cell types on Th cell function, and includes both autocrine and paracrine regulation whereas in cloned T cell tests for the cytokines produced by these clones is limited to autocrine regulation. Because our observations were based on cytokine production by all the cells circulating in the peripheral blood and not on clonal isolation, we decided to identify the cytokine patterns observed as type 1 (mainly CMI-inducing), and type 2 (mainly humoral immunity-inducing).[22,23] Because we defined cytokines on a functional basis, we could enlarge the group of type 1 cytokines to include IL-12 and, probably, IL-15 (all cytokines that mainly stimulate CMI, but are not mainly or exclusively produced by clones of CD4+ T lymphocytes) in addition to IL-2 and IFN-γ. Similarly, IL-6, IL-10 and probably IL-13 were considered to be type 2 cytokines even if they are produced by cells other than CD4+ T lymphocytes, as their main effect is that of stimulating B cell activity and antibody generation (see Table 2).

To measure IL-4, we stimulated PBMC of HIV+ asymptomatic donors *in vitro* with PHA for 48 to 72 h, as suggested by kinetic studies, and we evaluated the amount of IL-4 present in the supernatants using a cell line (a kind gift of Dr William Paul, NIAID, NIH) whose growth is dependent on IL-4. (To simplify the assay, we are now measuring IL-4 using commercially available ELISA kits.) To analyse the production of IL-4 at the molecular level, we also quantified (in collaboration with Dr Thomas Wynn, NIAID, NIH) the expression of IL-4 mRNA in the PBMC of the same patients. We observed that IL-4 production was greatly augmented in PHA-stimulated supernatants of HIV+ individuals as compared to HIV− controls, and that the increased IL-4 production was most likely to be observed in the subset of HIV+ asymptomatic patients showing defective IL-2 generation in response to recall antigens.[24] Similarly, IL-4 mRNA was detected in PHA stimulated PBMC of HIV+ individuals, but not in unstimulated PBMC of the same individuals, or in HIV− controls.[24] Even more important, IL-4 neutralizing antibodies were capable to restore *in vitro* antigen-stimulated proliferation and IL-2 production in the majority of patients secreting high amounts of IL-4.[24]

Because IL-10 was described as able to suppress the secretion of type 1 cytokines and CMI even more powerfully than IL-4, we next decided to measure IL-10 production in HIV+ asymptomatic patients. Similarly to the methods used to generate IL-4, we stimulated PBMC *in vitro* with PHA for 48 h; the amount of IL-10 produced was measured using commercially available ELISA kits, and IL-10 mRNA was quantified using PCR methods. We observed that IL-10 production was augmented in HIV+ asymptomatic patients showing defective IL-2 production, and that the patients with the most severe defective IL-2 production (i.e. inability to generate IL-2 even in response to PHA) generated the highest quantities of IL-10.[25] By analogy to what was observed for neutralizing antibodies to IL-4, *in vitro* antigen-stimulated proliferation and IL-2 production could be restored in the majority of these patients by neutralizing antibodies to IL-10.[25] Similarly, we could restore *in vitro* antigen-stimulated proliferation and IL-2 production, as well as mitogen-stimulated IFN-γ production, by IL-12,[26] a prototypical type-1 cytokine described by Chehimi *et al.* as defective, even in the earlier phases of HIV infection.[27] Thus, we proposed that HIV infection is associated with the progressive impairment of type 1 cytokine production, and the progressive augmentation of type 2 cytokine secretion.[22,23] It is important to underline that these events are observed in the asymptomatic phase of HIV infection, before the development of full blown disease. These data, confirmed by numerous other authors,[28–37] are the experimental basis of the type-1/type-2 hypothesis of HIV infection.

Because cytokine secretion is associated with a quantity of diverse biological effects which are readily measurable, we suggest that the list of these effects, which are frequently observed in HIV+ patients and are presented in Table 3, strongly support the hypothesis of a profound cytokine imbalance in HIV infection. Thus, the impairment of delayed type hypersensitivity reaction is secondary to defective IFN-γ, IL-2 and IL-12 production, whereas hyper-IgE is secondary to the augmented production of IL-4, and hypereosinophilia is secondary to the augmented pro-

Table 3. Unfavourable prognostic signs in HIV infection

Decreased production of type 1 cytokines (IFN-γ, IL-2, IL-12)
Increased production of type 2 cytokines (IL-4, IL-5, IL-6, IL-10)
Reduced delayed type hypersensitivity (DTH) reactions
IL-4-driven hyper-IgE
IL-5-driven hypereosinophilia

duction of IL-5. All these parameters are predictors of poor prognosis in HIV infection.[38–46] Interestingly, it has recently become evident that defective *in vitro* antigen- and mitogen-stimulated IL-2 production is predictive for subsequent reduction in CD4 counts, the time to acquired immunodeficiency syndrome (AIDS), and time to death.[47]

Different patterns of disease progression in HIV infection

Higher CD4 counts, better preserved IL-2 secretion, and the absence of the clinical signs of type 1/type 2 cytokine imbalance listed in Table 3 are associated with a lack of progression of HIV infection. It has recently become evident that, although the vast majority of HIV+ individuals progress to AIDS, a minority of HIV+ patients exists that does not develop AIDs or show a critical reduction in CD4 T cells, despite long-lasting infection with HIV.[48] These patients have recently been defined long-term non-progressors (LTNP). Different groups have focused on diverse aspects of this phenomenon, the solution of which holds the possible key to a cure for AIDS. Two biological interpretations can be offered in the attempt to explain long-lasting HIV infection without AIDS. Thus, these patients may have been infected by a defective, less pathogenic HIV variant, or these patients may have a stronger immune response, capable of keeping HIV under control. More likely, a strong immune response is preventing HIV from becoming frankly virulent in these individuals. In support of this hypothesis are the findings by Ho and colleagues, and Fauci and colleagues indicating that strong HIV-specific CTL were observed in two groups of LTNP.[49,50] Additionally, it was recently shown that: (1) preserved T cell function correlates with stable CD4 counts;[51] (2) disease-free survival correlates with *gag*-specific cytotoxic T lymphocyte activity;[52,53] and (3) HIV infected chimpanzees become seropositive but do not progress toward AIDS.[54] Finally, it has recently been verified that neither a type 1-to-type 2 cytokine shift nor programmed cell death are present in HIV+ chimpanzees.[55,56]

We have recently analysed (in collaboration with the Clinica delle Malattie Infettive, Università di Milano) cytokine production in a group of LTNP, and compared the cytokine profile with that observed in a group of patients with progressive HIV infection. As we expected, a strong type 1/weak type 2 cytokine profile was observed in the LTNP, whereas a symmetrically opposite weak type 1/strong type 2 cytokine profile was observed in patients with progressive HIV infection.[57] Interestingly, a significantly increased percentage of CD4+/CD7− T lymphocytes (as remembered above, CD4+/CD7− T lymphocytes predominantly secrete type 2 cytokines) was observed in the individuals with progressive HIV infection, but not in the LTNP.[57]

We have performed (in collaboration with the Cattedra di Pediatria IV, Università di Milano) a second study in a cohort of vertically infected children with different patterns of disease progression. In fact, HIV vertical infection follows a bimodal pattern according to which 20% of vertically infected children develop AIDS and eventually die within the first year of life, whereas the remainder develop AIDS at a constant rate per year, reaching the median at about 5 years after birth.[58] We have thus studied a group of vertically infected HIV+ children who did not develop AIDS within the first year of life, and we identified two subsets of children of comparable age, the first one of which was asymptomatic, while the second one showed severe signs of HIV disease. The results indicated that, despite the presence of severe defects in type 1 cytokine production in both groups of children, a greatly increased type 2 cytokine secretion was characteristic of the symptomatic, but not of the asymptomatic children.[43] Interestingly, a significant association with hyper-IgE (the production of which is stimulated by IL-4 secretion) was observed in the paediatric cohort.[43] Thus, we suggest that a strong type 1/weak type 2 cytokine production is associated with delayed (or absent) progression of HIV infection to AIDS.

Susceptibility of T lymphocytes to programmed cell death (PCD) is differentially regulated by type 1 and type 2 cytokines

It has been observed that PCD is increased in HIV infection, and it was thus suggested that PCD could be one of the mechanism(s) primarily responsible for CD4 depletion in the progression to AIDS.[59,60] HIV+ lymphocytes undergo PCD in unstimulated conditions, and much more so when stimulated with mitogens.[60,61] One of the

major differences between HIV+ and HIV− lymphocytes is that whereas only mitogen-activated HIV− lymphocytes (blasts) will undergo PCD upon a second mitogenic restimulation, even resting (unstimulated) HIV+ PBMC will undergo PCD upon stimulation. Therefore, two serial *in vitro* stimuli are needed to induce PCD in HIV− lymphocytes, whereas a single *in vitro* stimulation will provoke PCD in HIV+ lymphocytes, suggesting that in HIV infection lymphocytes are preactivated *in vivo* to undergo PCD upon *in vitro* restimulation.[60,61]

Mitogenic stimulation will activate CD4 and CD8 lymphocytes via T cell receptor-mediated stimulation, inducing PCD in both subsets of T lymphocytes. It was recently observed that PCD is differentially regulated by type 1 and type 2 cytokines.[61] Thus, PCD was prevented *in vitro* by IFN-γ, IL-2 and IL-12[61] whereas PCD was not prevented, or was augmented, by IL-4 and IL-10.[61] Even more relevant was the observation that PCD could be prevented by neutralizing antibodies to type 2 cytokines, but could not be prevented, or was even raised by neutralization of type 1 cytokines.[62] We have recently observed that the selective stimulation of CD4+ lymphocytes by recall antigens will induce PCD exclusively in the CD4 subset, a situation that more closely resembles that observed *in vivo*. Even in this situation, PCD was oppositely modulated by type 1 and type 2 cytokines, or by the neutralization of type 1 or type 2 cytokines. Additionally, we verified that PCD is effected by lymphotoxin, and that lymphotoxin is responsible for a soluble-factor mediated amplifying loop which causes PCD in innocent-bystander lymphocytes. Finally, the PCD-inducing effect of lymphotoxin is differentially influenced by type 1 and type 2 cytokines (M. Clerici *et al.*, submitted). We suggest that the impaired production of type 1 cytokines and the augmented generation of type 2 cytokines characteristic of HIV infection results in the destruction of CD4 lymphocytes, which is increased by type 2 cytokines and mediated by lymphotoxin. Thus, antigen stimulation of HIV+ lymphocytes in the presence of abnormally low concentrations of IFN-γ, IL-2 and IL-12, and of abnormally elevated concentrations of IL-4 and IL-10 results in the induction of PCD, instead of the induction of T cell proliferation.

Virologic and immunologic correlates of poor prognosis are associated

Finally, it was suggested that the development of AIDS is secondary to the emergence of an HIV phenotype with a rapid/high replication rate, a tropism for different cell lines, and the capacity to induce the formation of syncytia *in vitro*.[62–67] To verify whether the isolation of syncytium-inducing HIV is correlated with the type 1-to-type 2 cytokine shift, we have analysed virologic and immunologic parameters in two groups of HIV vertically infected children of comparable age who have or have not progressed to AIDS. We observed that progression to AIDS in paediatric HIV infection is associated with isolation of HIV SI variants and increased production of the type 2 cytokines IL-4 and IL-10. Additionally, we observed that these two parameters are statistically associated and that extensive CD4 loss is associated both with the isolation of SI variants and increased IL-4 production (M. Clerici *et al.*, submitted). These recent data indicate that the virologic and immunologic parameters characteristic of advanced HIV infection are strictly associated, and strongly support a virologic–immunologic pathogenesis leading the appearance of AIDS.

Conclusions

We suggest that the complex qualitative alterations observed in HIV infection are responsible for the progression of HIV disease to AIDS, and that the dramatic reduction of CD4 T lymphocytes which is the hallmark of this disease is mainly secondary to phenomena of PCD that is increased by a type 1/type 2 cytokine imbalance. Thus, we suggest that every therapeutic approach to HIV infection should consider the necessity to restore the normal functionality of the immune system. These approaches could at least theoretically be based on the utilization of: (1) type 1 cytokines; (2) antibodies neutralizing type 2 cytokines; or (3) pharmacological compounds aimed at the selective stimulation of type 1 cytokine secretion and subsequent augmentation of CMI. It is important to notice that the results of a first clinical trial based on the utilization of IL-2 have shown significant improvement in CD4 counts (possibly via the prevention of IL-2-induced PCD).[68] Thus, we strongly favour approaches based on the restoration of normal cytokine production in the therapy for HIV infection, as we believe that a strong CMI is associated with better prognosis, and will ultimately be more capable of controlling HIV replication and disease progression.

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