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COMMENTARY

Chronic Immune Stimulation, Oxidative Stress, and Apoptosis in HIV Infection

Gabriele Baier-Bitterlich, Dietmar Fuchs and Helmut Wachter

INSTITUTE FOR MEDICAL CHEMISTRY AND BIOCHEMISTRY UNIVERSITY OF INNSBRUCK, A-6020 INNSBRUCK, AUSTRIA

ABSTRACT. Infection with the human immunodeficiency virus (HIV) is accompanied by a decrease in $CD4^+$ T cell numbers and the ultimate disruption of immunological functions. In sera of infected patients, elevated levels of interferon-γ are detected, which is indicative of an activated TH1-type immune response. T-cell-derived interferon-γ leads to the expression of various proinflammatory cytokines and enhanced macrophage capacity to secrete reactive oxygen intermediates. In addition, interferon-γ is the major stimulator for the biosynthesis of neopterin and its reduced form, 7,8-dihydroneopterin. Neopterin is known as a sensitive immune activation marker in clinical laboratory diagnosis. Recent data implied a potential role of neopterin derivatives in oxygen free-radical-mediated processes, e.g. high concentrations of 7,8-dihydroneopterin were found to interfere with the oxidant–antioxidant balance, and may lead to apoptosis of human cells. In addition, 7,8-dihydroneopterin was found to be effective in the activation of redox-sensitive transcription factors and in the induction of HIV-1 gene expression. In this commentary, we describe our current view as to how neopterin derivatives, in concert with cytokines and reactive oxygen intermediates, may lead the way to the final destruction of the cellular immune system. BIOCHEM PHARMACOL 53;6:755–763, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. human immunodeficiency virus; AIDS; oxidative stress; apoptosis; neopterin; 7,8-dihydroneopterin

For the establishment of a productive infection with HIV,† the activation of the host cell appears crucial [1–4]. In addition to human replication, multiple events are hereby initiated, including the production of cytokines, i.e. interferon-γ [5], and host cell death [6]. T-cell-derived interferon-γ, affiliated with activated cell-mediated immunity, leads to the expression of proinflammatory cytokines and enhanced macrophage capacity to secrete reactive oxygen intermediates [7, 8]. In addition, IFN-γ is the major stimulator for the biosynthesis of neopterin and 7,8-dihydroneopterin from GTP [9–11]. *In vivo*, the production of neopterin closely correlates with IFN-γ concentrations, and elevated levels of neopterin and 7,8-dihydroneopterin have been detected in many diseases, such as infections or autoimmune disorders [11–15]. Recent results propounded a potential role for neopterin derivatives in oxygen radical-mediated processes [16–20], and 7,8-dihydroneopterin and neopterin were shown to be involved in redox signaling, leading to T-cell apoptosis and the activation of transcription factors NF-κB and AP-1 [21, 22]. Lately, it became more apparent that programmed cell death (apoptosis) and latent virus activation may be linked to "oxidative stress" in HIV infection [23–26]. Thus, neopterin derivatives may directly interfere with the ability of the cells to maintain an appropriate oxidant–antioxidant balance and the interaction between HIV replication and activated cell-mediated immunity. In HIV infection, a desired positive function of immune response is apparently overruled by negative side-effects in the chronic situation including oxidative stress, consequently leading to severe immune deficiency.

CHRONIC IMMUNE STIMULATION

During the course of infection with HIV [1, 27], three major phases may be distinguished [5, 28–30]. Within a few weeks post-infection, extensive viremia occurs, accompanied by large numbers of infected $CD4^+$ T cells and acute symptoms arising. During this time, an activation of the cellular immune system can be observed very often, e.g. by increased neopterin, which is secreted by IFN-γ activated monocytes/macrophages [12]. When humoral and cellular immune responses to HIV become established, the amount of circulating virus declines [31, 32], leading to a subclinical phase that is characterized by a lack of symptoms, low levels

* Corresponding author: Dr. Gabriele Baier-Bitterlich, Institute for Medical Chemistry and Biochemistry, University of Innsbruck, Fritz Pregl Str. 3, A-6020 Innsbruck, Austria. Tel. 43-512-507-3516; FAX 43-512-507-2865.

† *Abbreviations:* HIV, human immunodeficiency virus; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; ROI, reactive oxygen intermediate; IL, interleukin; NF-κB, nuclear factor κB; and AP-1, activation protein.

of virus and circulating infected cells, and moderately declining levels of $CD4^+$ T cells (Fig. 1). Recently, however, it became more obvious that constant virus production in this asymptomatic phase is still taking place and represents one of the most important forces driving AIDS pathogenesis [33–35]. Interestingly, in the majority of asymptomatic patients, neopterin is also increased all the time. On the average, 10 years post-infection AIDS develops, and virus and infected cells drastically reappear in the periphery, associated with a sharp decrease in $CD4^+$ T-cell numbers and ultimate disruption of immunological functions [5, 29, 30, 36, 37]. Early functional defects of cell-mediated immunity involve mainly the $CD4^+$ TH1-cell population. TH1 cells are differentiated $CD4^+$ T-helper cells that, upon stimulation, secrete IL-2 and IFN-γ, promote macrophage activation, and induce delayed-type hypersensitivity reactions and cell-mediated immunity, whereas TH2 cells secrete cytokines IL-4, IL-5, and IL-10, favoring B-cell activation [38–42], and lead to a down-regulation of delayed-type hypersensitivity reactions [5, 36]. This loss of $CD4^+$ TH1-cell function was proposed to be due to a progressive shift of $CD4^+$ T-cell development from TH1 to TH2, leading to a deprivation of IL-2 and IFN-γ production, accompanied by

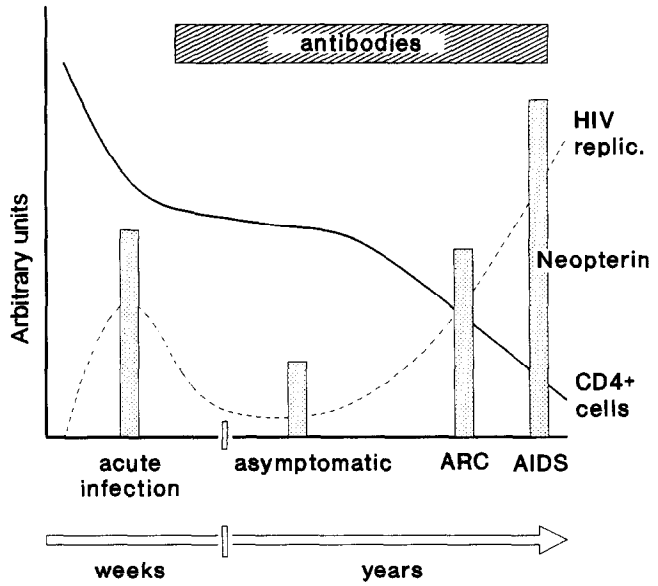

FIG. 1. Schematic changes of neopterin during the course of HIV infection. Within a few weeks post-infection, large numbers of infected CD4-positive T cells, viremia, and acute symptoms may be observed in patients, accompanied by an activation of the cellular immune system, as reflected, for example, by rising concentrations of neopterin. Upon the onset of humoral and cellular immune responses, neopterin concentrations decline as does the amount of circulating virus. The subclinical phase, characterized by a lack of symptoms, moderately declining numbers of $CD4^+$ T cells, and low levels of circulating infected cells, is associated with moderately elevated neopterin concentrations, indicating continuous virus production in many cases. On the average, 10 years post-infection AIDS develops, associated with a sharp decline in $CD4^+$ T-cell numbers and ultimate disruption of virtually all immunological functions characterized by high levels of neopterin.

increases in IL-4 and IL-10 secretion [43, 44]. This assumption stands in contrast to our results, which demonstrate enhanced IFN-γ levels in sera of patients with HIV infection, indicative of activated TH1-type immune response [45]. Similarly, enhanced expression of IFN-γ was demonstrated in lymph node T cells including $CD4^+$ cells of patients throughout the course of the disease [46]. Data of other investigators also support the view that there is no general decline of all TH1-type cytokines in HIV infection. Ameisen *et al.* [47] have studied the TH1/TH2 cytokine secretion profile in HIV-infected persons, with respect to the investigation of a possible role of these cytokines in programmed cell death (apoptosis). Data indicated that stimuli that initiated apoptosis in T cells from HIV-infected persons led to comparable *in vitro* levels of IL-2 and IFN-γ in HIV-infected persons and healthy controls. In addition, no significant IL-4 or IL-5 secretion was detected following *in vitro* stimulation, and IL-10 secretion was similar in activated T cells from HIV-infected persons and controls. These findings are consistent with recent results from cytokine messenger RNA analysis *in vitro* in the lymph nodes of HIV-infected persons [48]. It was further suggested [47] that in HIV-infected persons IL-2 is expressed solely by $CD4^+$ T cells, whereas IFN-γ is excreted mainly by $CD8^+$ T cells. In contrast, others [49] have shown that IFN-γ mRNA was increased markedly, preferentially in the $CD4^+$ T-cell subset of HIV-seropositive patients. The contradictory results regarding the producer cells of IFN-γ, however, do not alter the immunological consequence of an enhanced expression of this cytokine, namely to direct T-cell activation towards the TH1-type cells. Along this line, doubt has been raised over the biological activity of endogenous IFN-γ in patients; likewise, IFN-γ therapy in AIDS patients was not associated with improvement of the immunological status of these patients [50]. Various clinical studies [45, 51], however, showed that concentrations of neopterin are correlated to IFN-γ, which indicates that biological activity of IFN-γ is capable of inducing neopterin production in macrophages. In addition, various clinical findings support the concept that IFN-γ is indeed involved in the pathology of HIV infection, e.g. an association between immune activation, reflected by elevated IFN-γ and neopterin levels, and decreased hemoglobin levels was detected in patients with HIV infection [52]. Likewise, weight loss and cachexia are associated with high neopterin levels in patients. IFN-γ, especially in association with TNF, is known to induce anemia and cachexia in animal models and in humans.

T-cell-derived IFN-γ, affiliated with activated cell-mediated immunity, leads to the expression of proinflammatory cytokines and enhances macrophage capacity to secrete ROIs [7, 8]. In addition, IFN-γ is the major stimulator for the biosynthesis of neopterin and its reduced form, 7,8-dihydroneopterin, from GTP [9, 10, 53]. In common, acute viral infections are accompanied by high neopterin levels that reflect activated cell-mediated immunity and, thus,

TH1-type immune response [12]. In contrast, in the acute phase of bacterial infections, TH2-type immune response is activated; in this situation, comparably low neopterin values are observed and neopterin levels can even be used to discriminate bacterial from viral infections [54]. Only in chronic bacterial infections may concentrations of neopterin rise [54], possibly based on the interaction of a multitude of cytokines such as IL-1 and TNF-α, which at some point may also activate the TH1 cell compartment and trigger release of IFN-γ.

Neopterin and 7,8-dihydroneopterin are small molecules (253 and 255 Da) that are excreted in constant proportions with a ratio of aromatic neopterin to total (aromatic plus oxidizable) neopterin for 1:3 for urine and 1:2 for serum obtained from venous blood samples [15, 55]; in arterial blood and cerebrospinal fluid, a higher proportion of 7,8-dihydroneopterin was found [55, 56]. Due to the chemical instability of the dihydro forms, collection and storage of samples for measurements of 7,8-dihydroneopterin are critical and problematic for large-scale handling in the clinical routine [15]. Hence, for diagnostic purposes only the more stable neopterin is being quantified. In serum, neopterin is usually determined by immunoassays with concentrations averaging 5.3 ± 2.7 nmol/L in healthy adults [11, 57], and, since neopterin is cleared constantly by the kidneys [58], urine measurements of neopterin derivatives can also be performed easily by examining samples by high-pressure liquid chromatography [11, 57]. Hence, it is necessary to relate neopterin concentrations to urine creatinine levels measured in parallel to take fluctuations of urine densities into account, and results are expressed in micromoles of neopterin per mole of creatinine.

Human monocytes/macrophages constitute the most relevant source of neopterin when activated with IFN-γ, or with an at least 1000-fold higher concentration of IFN-α [9]; lipopolysaccharide and TNFα are able to superinduce IFN-γ-mediated neopterin production [59]. The key enzyme of the biosynthetic pathway for the formation of neopterin is GTP cyclohydrolase I, which cleaves the purine GTP to synthesize 7,8-dihydroneopterin triphosphate, which, in turn, is cleaved by phosphatases to neopterin and 7,8-dihydroneopterin [60], thus becoming detectable in cell culture supernatants and body fluids [11, 61]. In the late 1970s, high neopterin levels were detected in cancer patients and patients suffering from viral infections [13], which led to the assumption that neopterin excretion may be closely connected to the immune response of the host. This assumption was confirmed by subsequent findings of neopterin in mixed lymphocyte cultures of peripheral blood mononuclear cells [62].

In vivo, the secretion of neopterin closely correlates with IFN-γ concentrations [45, 51] and the activation of cell-mediated immunity, e.g. in virus infections including HIV infection, in certain types of cancer, and in autoimmune disorders [11, 14, 61]. Increased neopterin concentrations are found in asymptomatic HIV antibody seropositive individuals (e.g. over 90% of seropositive parenteral drug addicts), yet seronegative homosexuals, drug addicts, and hemophilia patients also may show increased neopterin levels [12]. Neopterin concentrations correlate significantly with the stages of HIV infection (Fig. 1). Patients with higher neopterin levels in a certain stage of the disease progress significantly more rapidly to higher stages than patients with lower neopterin levels, and AIDS patients with the highest neopterin levels are more likely to die earlier than other AIDs patients [63]. Interestingly, in the final stage of AIDS, extremely low numbers of T-lymphocytes ($CD4^+$ cells) can induce the release of extremely high neopterin concentrations. This apparent paradox may be explained by a polyclonal stimulation of the remaining T-helper cells. One might even speculate that the amount of activated T cells in the final stage of AIDS still surpasses that of a normal immune stimulation [64].

PROGRAMMED CELL DEATH (APOPTOSIS)

Numerous mechanisms including immune suppression, hyperactivation and exhaustion of cells, and selective infection and destruction of memory T-helper cells have been made accountable for the early loss of T-helper cell response "*in vitro*" to major histocompatibility complex (MHC) class-II restricted recall antigens and to specific polyclonal activators such as anti-CD3 antibodies [65]. Later it became apparent that the loss of $CD4^+$ T cells in HIV infection is associated with incomplete lymphocyte activation that does not result in cell proliferation, but rather in cell death by a process that is physiologically crucial for the proper development and homeostasis of many tissues, and is called apoptosis [6, 66–68]. In addition to being observed in $CD4^+$ T cells, anergy and apoptosis were also found in $CD8^+$ T cells from HIV-infected persons [69–72]. Early investigations of the pathologic mechanism underlying T-cell death in HIV-infected persons showed that cross-linking of the CD4 molecule by anti-CD4 antibodies or by the HIV-1 envelope protein can trigger apoptosis in human $CD4^+$ T cells [73–77]. Interestingly, Finkel *et al.* [78] observed that DNA fragmentation, one of the hallmarks of a certain type of programmed cell death, is rarely detected in productively infected cells but rather in uninfected "bystander cells." Among the putative deficient biochemical signaling events associated with negative signaling via the CD4 coreceptor, diminished Ca^{2+} mobilization, inositol phospholipid metabolism, and mitogen-activated protein kinase activation have been observed [79–83]. Recently [84–86], an increased expression of the apoptosis-related membrane antigen Fas (CD95, APO-1), a member of the nerve growth factor/tumor necrosis factor receptor family [87], on $CD4^+$ T cells was described in HIV-positive individuals. This up-regulation of Fas may be initiated via cross-linking of CD4 molecules, and IFN-γ and tumor necrosis factor-α may augment Fas expression in a dose-dependent fashion [88]. Fas-induced apoptosis was

shown recently to be mediated via a ceramide-initiated Ras signaling pathway [89]. T-cell apoptosis may also be induced by the HIV-1 transactivator protein Tat [90–94]. Tat-induced apoptosis possibly involves cyclin-dependent kinases [95], which fits with the observation that uninfected lymphocytes are abnormally activated in HIV-1-infected patients [5, 6, 95, 96]. The incomplete activation of lymphocytes, which may lead to cell death by apoptosis, is paralleled *in vivo* by an incomplete immune response as reflected by the secretion of IFN-γ without the company of IL-2 [49]. A similar functional defect of T-cells can be observed in other diseases such as cancer and autoimmune diseases as well [51]. It was further suggested that chronic activation of the immune system(s), which is observed during the course of HIV infection, may be the primary mechanism responsible for cellular depletion by apoptosis [97]. Moreover, IFN-γ possibly leads directly to severe immune dysfunction in patients with AIDS [98]. Indeed, treatment of HIV-infected patients with IFN-γ did not improve T-cell functional response *in vitro* [50].

As mentioned above, IFN-γ-activated monocytes/macrophages express TNF-α and IL-1, secrete neopterin and 7,8-dihydroneopterin, and enhance the capacity of macrophages to secrete reactive oxygen intermediates [7, 8]. Indeed, infection with HIV is associated with an increased production of numerous cytokines such as TNF-α and IL-6 whose target cells are either T cells or monocytes/macrophages or both [5]. To investigate the potential role of neopterin derivatives in cellular destruction, we have chosen U937 cells, a human histiocytic lymphoma cell line, which are known to undergo apoptosis after incubation with TNF-α [99–101]. Cells were preincubated with various concentrations of neopterin and 7,8-dihydroneopterin followed by activation with TNF-α. Then apoptosis was evaluated with various methods (ultrastructural analysis by electron microscopy, and by dUTP-FITC nick end labeling (terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling method) [102], and quantified by fluorescence-activated cell sorting analysis of propidium iodide-stained isolated nuclei. While increasing concentrations of neopterin did not show a significant effect, low concentrations of 7,8-dihydroneopterin (up to 300 μM) appeared to inhibit apoptosis mediated by TNF-α, whereas higher concentrations (5 mM) superinduced TNF-α-mediated apoptosis [20]. As will be discussed later, neopterin, in contrast to 7,8-dihydroneopterin, may require additional cofactors for effectiveness. Since T cells are obviously most prone to undergo apoptosis in HIV infection, we further tested cells of the T-lymphocyte lineage (Jurkat clone E6-1). Even in the absence of a costimulant such as TNF-α, 7,8-dihydroneopterin (5 mM) induced apoptosis in Jurkat cells. During a relatively short time period of 3.5 h, 67% of the cell population showed apoptotic characteristics. These results led us to the assumption that, in addition to being a useful marker for AIDS progression, neopterin derivatives may actively take part in the destruction of the immune system.

OXIDATIVE STRESS

Programmed cell death and latent virus activation may be linked intimately to "oxidative stress" caused by excessive production of ROIs and a resulting deficiency of antioxidant mechanisms in HIV infection [24]. Reactive oxygen species are produced continuously during activation of phagocytes as a defense mechanism against environmental pathogens (Fig. 2). They are equally important for the activation of T cells, e.g., in the initiation of antigen-specific immune responses. Antioxidants such as cysteine and glutathione (GSH) may antagonize this process [25, 26]. The high level of antigenic and cytokine activity in HIV/AIDS results in an elevated production of ROIs (i.e. superoxides, hydrogen peroxide, and hydroxyl radicals). Antioxidant defenses such as important reactive-oxygen scavenger enzymes, manganese superoxide dismutase and catalase, may thereby be overwhelmed [103, 104]. Likewise, HIV-infected patients often have decreased plasma cystine and cysteine concentrations, decreased intracellular GSH, an important radical scavenger, and elevated plasma concentrations of glutamate, which is a competitive inhibitor of the membrane transport of cystine [105–108]. Elevated glutamate levels and decreased cystine and cysteine levels in HIV-infected persons could even be responsible for the selective decrease of $CD4^+$ T cells [25, 109].

Neopterin may serve as an indirect marker for oxidative stress, since neopterin secretion by monocytes/macrophages correlates to their oxidative burst capacity [110]. Moreover neopterin derivatives may directly interfere with the intracellular redox balance because a potential function of neopterin and 7,8-dihydroneopterin in oxygen-radical-mediated-processes was demonstrated [20]. In a luminol assay, neopterin enhanced chloramine-T and hydrogen peroxide-mediated chemiluminescence, while 7,8-dihydroneopterin was shown to be a potent scavenger. In addition, neopterin was demonstrated to potentiate toxicity of chloramine-T against bacteria [16]. Later it was illustrated [17] that neopterin enhances hydrogen peroxide effects only in the presence of iron chelator complexes at neutral or slightly alkaline pH and that 7,8-dihydroneopterin quenches hydrogen peroxide-induced chemiluminescence only at low concentrations [20, 111], while at a high concentration (5 mM) 7,8-dihydroneopterin acts as an enhancer. As demonstrated in cell culture assays, this concentration in combination with TNF-α superinduces the intracellular formation of reactive oxygen species [20]. Based on these data, it is likely that the induction of apoptosis by 7,8-dihydroneopterin and TNF-α (see above) is due to interference with the intracellular redox balance of U937 monocyte-like cells and Jurkat T-

[1] Baier-Bitterlich G, Baier G, Fuchs D, Böck G, Hausen A, Utermann G, Pavelka M and Wachter H, *Oncogene* (in press).

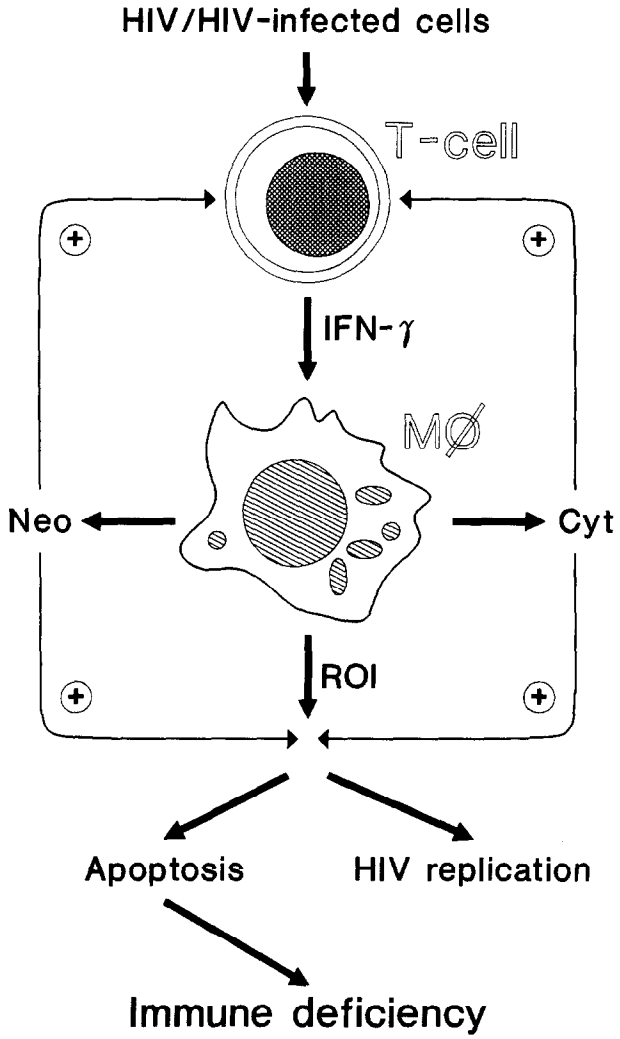

FIG. 2. Proposed role of 7,8-dihydroneopterin in HIV infection. HIV infection leads to the activation of the immune system. Hence, T cells secrete IFN-γ, which leads to the expression of various proinflammatory cytokines (Cyt) such as TNF-α and to an enhancing of the macrophage capacity to secrete ROIs. In parallel, human macrophages activated by IFN-γ secrete neopterin and 7,8-dihydroneopterin (Neo). Hence, neopterin derivatives, i.e. 7,8-dihydroneopterin, and neopterin may synergize with ROIs and Cyt, i.e. TNF-α, in the induction of HIV-1 gene expression due to activation of NF-κB, an enhancer element of the HIV LTR promoter. Neopterin derivatives may up-regulate their own production by inducing IFN-γ. Similarly, proinflammatory Cyt secreted by monocytes/macrophages further enhance T-cell activation. In parallel, neopterin derivatives in concert with Cyt and ROIs may contribute to cell death by apoptosis and to the loss of immune competence.

lymphocytes. Indeed, apoptosis was down-regulated significantly by known antioxidants such as *N*-acetylcysteine. This substance has been shown to inhibit [112] the activation of NF-κB, a transcription factor for the expression of several immunologically important genes, including the IL-2 receptor α-chain and TNF-α, of the MHC genes, and the *c-fos* gene [113, 114]. NF-κB is also an important enhancer of the HIV promoter [115]. NF-κB is activated by a number of substances including IL-1, TNF-α, and the tumor promoter (12-O-tetradecanoylphorbol-13-acetate), that also induce ROI production [23, 25, 113, 114]. Along this line, hydrogen peroxide also shown to induce NF-κB [23]. We therefore were inclined to test the impact of 7,8-dihydroneopterin on the two redox-sensitive transcription factors, NF-κB and AP-1 [23, 116–118]. After transfection of Jurkat T cells with promoter reporter-gene constructs containing wild-type or mutated NF-κB or AP-1 binding sites [21], they were incubated with 7,8-dihydroneopterin, neopterin, and/or TNF-α. Increasing concentrations of 7,8-dihydroneopterin enhanced the specific activation of AP-1 CAT, which fits well with earlier data showing that neopterin derivatives induce *c-fos* gene expression [119]. A significant induction of transcriptional activation of NF-κB-CAT was only achieved by 7,8-dihydroneopterin in combination with TNF-α [21]. These data correspond to recent findings [94] that HIV-1 Tat potentiates TNF-induced NF-κB activation. Results are especially interesting in view of the fact that NF-κB is a central enhancer element of the HIV LTR promoter [115] and that AP-1 plays a central role in antigen-mediated activation of the HIV-2 LTR [120].

SUMMARY

Neopterin has long been known to be a potent marker for HIV progression [12, 121, 122[. Based on our recent findings that neopterin and 7,8-dihydroneopterin are potentially involved in oxygen free-radical-mediated processes [20, 21], we hypothesize that neopterin derivatives apparently join the line of agents and cytokines that directly influence the fate of cells in HIV infection. Infection with HIV is associated with an increasing production of cytokines [5] and neopterin [12]. They may directly affect virus replication by disturbing the redox balance and activating redox-sensitive transcription factors [21]. In response to free radical activity supposedly produced in excess in patients with HIV/AIDS monocytes/macrophages may enhance cytokine production, e.g. TNF [24]. Neopterin derivatives may play an important role in causing a further increase in the levels of cytokines and ROIs by: (1) providing an amplification loop that feeds back to excite further production of virus by activating redox-sensitive transcription factors that are enhancer elements of the HIV promoter, and (2) inducing the production of IFN-γ by activated T-cells [123]. In addition, other sources of ROIs including products of peroxidation and inflammatory responses, cofactors such as mycoplasma, and nitric oxide [24] may aggravate this process that leads along with several other mechanisms [5] to the final destruction of the immune system in HIV infection. Thus, in HIV infection a desired positive function of immune activation is apparently overruled by its negative side-effects in the chronic situation, including oxidative stress, consequently leading to severe immune deficiency.

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