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Cytokine associated neopterin response of peripheral blood mononuclear cells to in vitro Epstein–Barr virus transformation process of B lymphocytes*

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Aim: The Epstein–Barr Virus (EBV) is a human herpes virus that infects B lymphocytes latently and immortalises the cells due to transformation. EBV infection is asymptomatic in childhood, while it may cause a self-limiting lymphoproliferative disorder in adolescence. This study aimed to show the association between in vitro transformation of B lymphocytes via EBV and the levels of neopterin with different accompanying cytokine responses.

Materials and methods: Mononuclear cells were isolated from peripheral blood samples of healthy donors and transformed with an EBV secreting cell-line, B95-8. Neopterin, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and interleukin (IL)-6 levels were measured in culture supernatants using enzyme immunoassay.

Results: Neopterin levels increased dependent on time and independent of EBV transformation. In transformed cell cultures, TNF-α and IFN-γ levels reached their peak in the first week and decreased in the third week; however there were no significant differences between the 3 weeks. IL-6 reached its maximum level in the third week.

Conclusion: According to these results, neopterin levels, which increased with time and independently of EBV transformation, may be a helpful marker for evaluating the acute response to viral infection, but not for B lymphocyte transformation

Key words: B lymphocyte transformation, EBV, in vitro, neopterin

1. Introduction

The Epstein–Barr Virus (EBV), a herpes virus that causes persistent infection in the host (1), can infect and transform human B lymphocytes and bring them into an immortal stage (2). B lymphocytes generate the main reservoir for EBV in human organisms. The virus may attach its glycoprotein gp350/220 to the cell surface via the complement receptor CD2/CD21, and has the ability to transform these cells. Autocrine growth factors, such as interleukin (IL)-6, are crucial for these transformed cells to maintain their immortal stage (3). Interferon (IFN)-γ and IL-6 levels increase in acute self-limiting infectious mononucleosis (IM), but in the other forms of EBV mediated diseases, such as chronic-active IM and X-linked lymphoproliferative disorder (XLPD), a specific IL-6 increase is not as significant as in acute forms (4). These varying cytokine responses, which are dependent on the type and stage of the disease, show that the relation between host and EBV mediated lymphoblastic transformation is linked to many different parameters. Cytokine release may change depending on the stage of infection (acute or chronic) and also on the type of transformation, proteins expressed during the process, or the growing tumour. Another molecule related to the progression of viral infections is neopterin, which is a pteridin molecule synthesised from guanosine triphosphate (GTP) (5). Since monitoring its levels may be a guide to rate the progression of disease, neopterin levels also have some predictive rates in allograft rejection, HIV infection, in some malignancies, and also in neurologic and cardiovascular disorders (6). In immune mediated oxidative stress, neopterin levels may also increase depending on the intensity of the antioxidative response (7).

B95-8, a peripheral blood lymphocytic cell line, is the most frequently used cell line for transformation of B lymphocytes via EBV in vitro (8). After transformation the morphologic differences in cell culture start with the attachment of the virus to the cell surface via the CD21 molecule and will be prominent after 3–4 days. The most important morphologic signs of transformation in an

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actively growing cell culture are cellular clumps attaching to the surface of the culture flask, and they are accepted as the first alteration that may be seen with the naked eye. It is reported that for a full transformation of cell culture a time period of 6–8 weeks is necessary (9). Transformed cells lose their contact inhibition and they proliferate on one another, causing a composition of thick cell layers. Attaching to solid surfaces is another capability that a transformed cell loses in the culture (9).

This study aimed to show the interaction between in vitro EBV transformation and neopterin, which is a marker for activity of cellular immunity and accompanying proinflammatory cytokine responses such as IFN-γ, tumour necrosis factor (TNF)-α, and IL-6.

2. Materials and methods

The following materials were purchased from commercial suppliers: fetal bovine serum (FBS) (Biochrom AG, Cat. No. S0115, Germany), RPMI-1640 (2.0 g/L $NaHCO_3$, stabile glutamine, low endotoxin) (Biochrom AG, Cat. No. FG1215, Germany), 0.9% NaCl (I.E. Ulagay Pharmaceuticals, Turkey), heparin (Nevaparin, 5000 IU/mL; Mustafa Nevzat Pharmaceuticals, Turkey), Ficoll-Hypaque (Histopaque-1077, Sigma Aldrich Co., Cat. No. 1077-1, USA), trypan blue (Applichem, C.I. 23850, CAS No. 72-57-1, Germany), phytohaemagglutinin (PHA-L) (Sigma Aldrich Co., No. L2769, USA), and lipopolysaccharide (LPS) (Sigma Aldrich Co., No. L-3755, USA).

2.1. B95-8 cell culture

B95-8 cell line was stored in liquid nitrogen prepared as described before by Coligan (10) and transferred to culture flasks containing RPMI-1640. Flasks had been kept in a 37 °C, 5% CO_2 water-jacketed incubator for 3 days until the cells achieved the optimum concentration.

2.2. Subjects and blood sampling

This study was approved by the Local Ethics Committee of the Gazi University Faculty of Medicine and was carried out in accordance with the Helsinki Declaration. A signed informed consent form was obtained from each participant.

In the first part of this study 8 healthy volunteers between the ages of 25 and 35 had their serum samples tested, first with immunofluorescence, a gold standard method for detecting the antibodies against the EBV capsid antigens including antiviral capsid antigen (VCA) immunoglobulin (Ig) G, antiEBV nuclear antigen (EBNA) IgG, and antiearly antigen (EA) IgG, and also antiVCA IgG avidity (Biochip Sequence EBV, Euroimmun Medizinische Labordiagnostica AG, Germany). A 20-mL venous blood sample was obtained from the antecubital vein of each volunteer. Peripheral blood mononuclear cell (PBMC) isolation (11) and transformation (12) were performed as described before by Coligan. Since there was no significant difference in the preliminary studies between the cultures containing 10% FBS or 2% AB Rh(+) human serum, we decided to continue the experiments with AB Rh (+) human serum. AB Rh (+) human serum was obtained from a 42-year-old healthy male donor, and was inactivated at 56 °C and filtered using a 0.2 μm microfilter. Isolated PBMCs were titrated in quadruplicate in 48-well cell culture plates, in a dilution of 500,000 cells per well in a RPMI-1640 medium with 2% AB Rh (+) human serum. There was 1 control set, and 2 sets were stimulated with PHA (with a final concentration of 10 μg/mL), and LPS (with a final concentration of 20 μg/mL). The last set was transformed by adding 10 μL of B95-8 culture supernatant. Samples from stimulated, nonstimulated, and transformed cell culture supernatants were obtained at 0, 12, 24, 48, and 72 h and from weeks 1, 2, and 3 and transferred to a 96-well flat-bottomed microplate and kept at –80 °C until the day of analysis.

2.3. Cytokine assays in cell culture supernatants

Neopterin, IFN-γ, and TNF-α levels were measured using commercial enzyme linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions (Neopterin ELISA, Demeditec Diagnostics GmbH, Germany; human TNF-α ELISA, human IFN-γ ELISA: BioSource International Inc., USA). According to the neopterin results, IL-6 levels were measured in the supernatants of the B95-8-transformed group at 12 h, when IL-6 started to increase, using a commercial ELISA kit according to the manufacturer's instructions (human IL-6 ELISA: BioSource International Inc., USA). Optic densities (ODs) developed after the study were measured using a 450 nm filter with an automatic ELISA reader, spectrophotometrically.

2.4. Statistical method

Neopterin and cytokine levels were calculated using Microstat software. Statistical analysis between groups was performed by one-way analysis of variance (ANOVA) with Bonferroni's posthoc test, using SPSS version 10.0. For all analysis, a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. EBV antibodies detected by immunofluorescence

Serum samples were inspected for antibodies against VCA, EBNA, and EA by immunofluorescence. None of the volunteers showed antiVCA IgM positivity. All of them were antiVCA IgG positive, 1 of them was antiEA IgG positive and 2 of them were antiEBNA IgG negative. AntiVCA IgG avidities were all high and positive, excluding a new infection history that had occurred in the previous few months.

3.2. Neopterin levels

After 12 h of cell culture peripheral blood mononuclear cells, which had been isolated from the blood samples of 8 healthy volunteers, attached to the flask bottom as monolayers (Figure 1). At the end of the first week EBV infected cells showed a stronger attachment than the others, suggesting a successful transformation (Figure 2). Neopterin levels increased in all groups, and at the end of week 3 it reached 40.53 nmol/L in the control group, 63.79 nmol/L in the PHA-stimulated group, 48.20 nmol/L in the LPS-stimulated group, and 65.34 nmol/L in the transformed group (Figure 3). In the transformed group mean neopterin levels in all 3 weeks when the transformation occurred were significantly different compared to all of the other time-points ($P < 0.05$), but there was no statistically significant difference between the 3 weeks ($P > 0.05$). When neopterin levels in cultures to which different stimulators were added were compared according to the time-points, there were significant differences between the control and all other stimulated and transformed groups ($P > 0.05$). Although PHA-stimulated cells did not show any significant difference, in transformed cell cultures neopterin levels were significantly different compared to those in cultures stimulated with LPS in weeks 1, 2, and 3 ($P > 0.05$). The correlation analysis for the weeks 1, 2, and 3 showed that neopterin increase was correlated with IFN-γ increase in weeks 1 and 3 ($P < 0.001$, Pearson = 0.720; $P < 0.05$; Pearson = 0.733, respectively), but the correlation in week 2 was insignificant ($P > 0.05$).

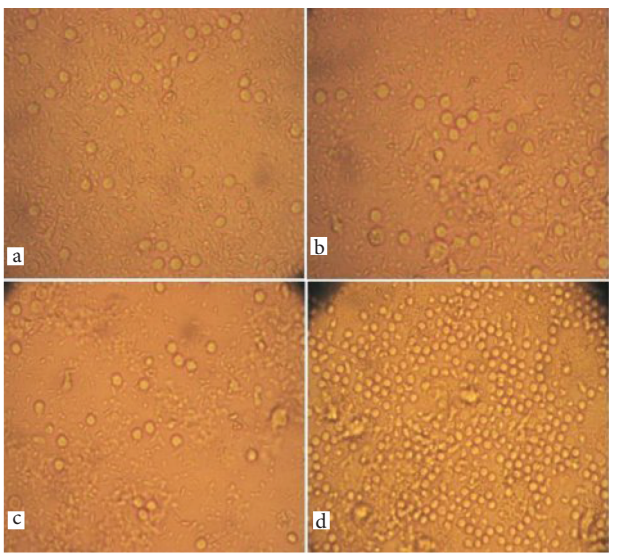

Figure 1. Time-dependent morphological changes in nonstimulated cell cultures: (a) 24 h, (b) 72 h, (c) week 1, (inverted microscope, 40×); (d) week 3 (inverted microscope, 20×).

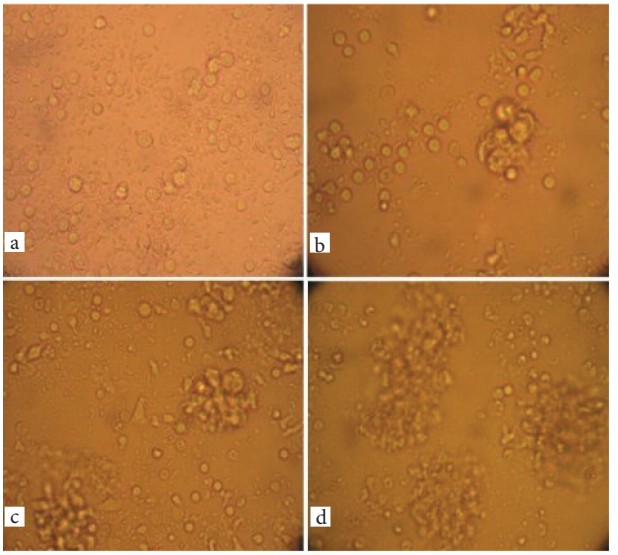

Figure 2. Morphological changes in cell cultures transformed by Epstein–Barr virus (EBV) secreting B95-8 cell culture supernatant: (a) 24 h, (b) 72 h, (c) week 1, (d) week 3 (inverted microscope, 40×).

3.3. Cytokine levels

In contrast to the undetectable levels in the control group, TNF-α increased in the PHA- and LPS-stimulated groups and reached its peak levels at 12 h (749.26 pg/mL and 701.43 pg/mL, respectively). In the transformed group, TNF-α started to increase at 72 h (386.94 pg/mL) and reached to its peak level in week 2 (397.18 pg/mL) (Figure 4). There was no significant difference among the mean TNF-α values in all 3 weeks ($P > 0.05$).

The IFN-γ level was undetectable at the 0 time-point and then started to increase and reached its peak at 72 h in the PHA-stimulated group (1932.00 pg/mL) and in week 1 in the LPS-stimulated group (681.67 pg/mL) (Figure 5). EBV transformed cells showed an IFN-γ increase at 72 h, and reached its peak level in week 2 (1345.46 pg/mL). When the results were compared using ANOVA there was no significant difference until 72 h ($P > 0.05$), and the mean values at these time-points were significantly different compared to the values in all 3 weeks ($P < 0.05$). Similar to TNF, there was no significant difference among the 3 weeks at all ($P > 0.05$). In transformed cell cultures both TNF-α and IFN-γ levels showed a noticeably different response. Cytokine levels started to increase in week 1, when transformation occurred, and proceeded for 3 weeks. This significant response suggested that the transformed cells have different cytokine responses compared to the unstimulated and nonspecifically stimulated cells.

In the transformed cell culture IL-6 reached its peak level in week 3 (646.07 pg/mL) (Figure 6). The difference between the 0 time-point and 24 h was statistically significant (P

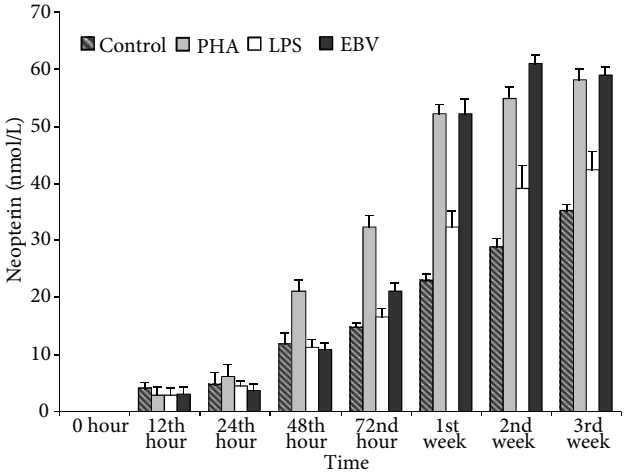

Figure 3. Neopterin levels in 2% AB Rh(+) human serum added nontransformed, stimulated, and transformed cell culture supernatants (PHA: phytohaemaglutinin, LPS: lipopolysaccharide). (Data are presented as mean ± standard error).

< 0.05) like the difference between 72 h and week 1, but, like the other cytokines, the difference among the 3 weeks was not significant ($P > 0.05$). Although IL-6 started to increase at 12 h, when the 12 h levels were compared for all groups, the control group and the transformed group were all significantly different to all other groups ($P < 0.05$), but there was no significant difference between the groups stimulated with PHA or LPS ($P > 0.05$).

4. Discussion

It is known that, especially in the chronic stage of infection, EBV causes certain pathologies, including some malignancies in vivo (1). In our study the 3-week duration was important in order to give an idea about the response against viral mechanisms in the chronic stage.

One of the significant characteristics of EBV is its transformational capability. Zerbini et al. (13) showed that EBV can transform most of the B lymphocytes in cord blood, as shown in adult B lymphocytes (14). In vitro infection of B lymphocytes with EBV may cause the development of B lymphoblastoid cell lines (LCLs) (15). In some cultures, where T lymphocytes have been depleted or physically inactivated, B cell transformation occurs more efficiently (16). In a previous study, where transformation was evaluated with LCL proliferation, transformed cells showed clumping, with increasing diameters and rising granularity (15). In our study, the cells attached to the flask bottom after 12 h (Figure 1) and transformed cells exhibited distinctively more clumping than others (Figure 2). When the cytokine responses of transformed cells were compared with those of stimulated cells or the control group, they showed a different characteristic.

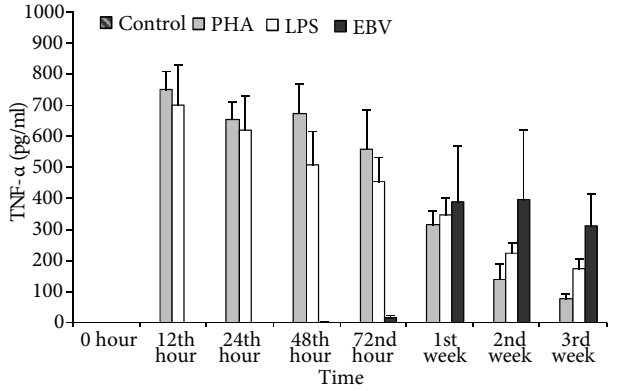

Figure 4. Tumour necrosis factor (TNF)-α levels in 2% AB Rh(+) human serum added nontransformed, stimulated, and transformed cell culture supernatants (PHA: phytohaemaglutinin, LPS: lipopolysaccharide). (Data are presented as mean ± standard error).

Our preliminary experiments showed that the neopterin levels also increased in cell cultures depending on time but not on stimulation or transformation. Since there was no significant difference in the preliminary studies between the cultures with either added 10% FBS or 2% AB Rh(+) human serum, we decided to continue the experiments with AB Rh(+) human serum. In the literature there are many studies in which 10% FBS was added to the cell culture medium (3). Whittingham et al. (17) compared FBS and human serum in their experiments and did not detect any difference between the 2 culture conditions. Since there was no significant difference in the increase in neopterin levels during 3 weeks (Figure 3), it is thought that this increase might be time dependent. EBV transformed cells showed a similar cytokine response, and this response was different from those of other unstimulated and also nontransformed cells.

The correlation analysis, which showed that neopterin increase was associated with IFN-γ increase in weeks 1 and 3 (Figure 3,5), supported the data that IFN-γ is the best known stimulator for neopterin production, both in vivo and in vitro. The time-dependent increase in the neopterin levels in transformed and nontransformed cell cultures could be related to another factor in the culture that might influence the cytokine response. A disadvantage of primary cell cultures is a condition called "culture shock". After isolation, 1%–10% of the cells may survive and others die due to the stress of the isolation process or their failure to adapt to the new environment (16). Culture shock may be induced cells in different ways, including oxidative stress. The response of the cells to reactive oxygen substances (ROS) may be associated with the type of cell, the amount of ROS, or to how they adapt to the culture (18). Although neopterin is known as a T cell activation marker, its level also increases due to the cellular

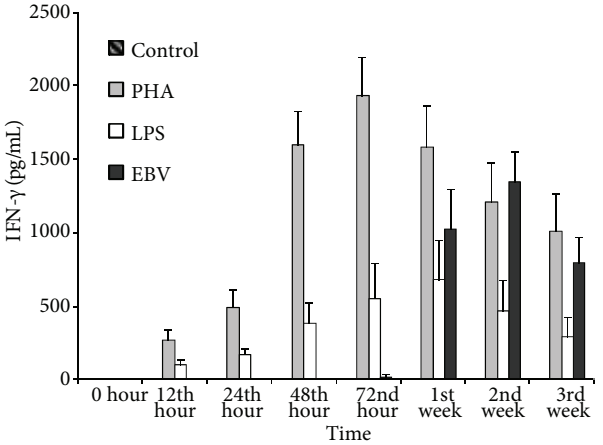

Figure 5. Interferon (IFN)-γ levels in 2% AB Rh(+) human serum added nontransformed, stimulated, and transformed cell culture supernatants (PHA: phytohaemagglutinin, LPS: lipopolysaccharide). (Data are presented as mean ± standard error).

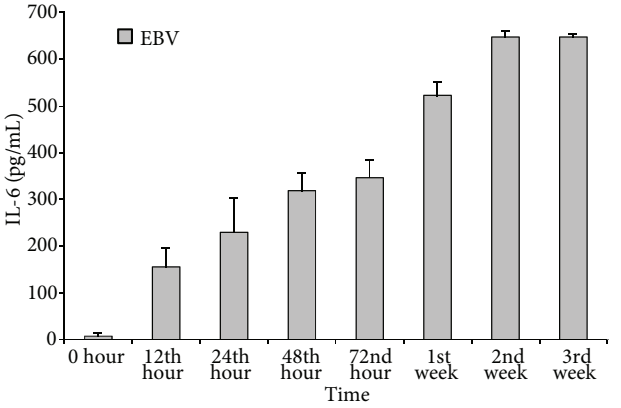

Figure 6. Interleukin (IL)-6 levels in 2% AB Rh(+) human serum added transformed cell culture supernatants. (Data are presented as mean ± standard error).

defence against oxidative stress. During cellular defence, mechanisms related to inflammatory and infectious conditions activate several immune cells, including monocytes and macrophages, and induce the production of reactive oxygen and nitrogen substrates (ROS/RNS) (19). ROS-induced oxidative stress causes various pathological conditions due to its alterative effects on cellular signalling systems, and also its destructive effects on lipids, proteins, and DNA (20). One effect of neopterin is to increase the cytotoxic effects of ROS and RNS as a response to several damaging pathogens. Neopterin level, secreted by activated monocytes/macrophages, is also related to hydrogen peroxide produced by the same cells (21). Although neopterin is not a scavenger molecule for free radicals, it works as a noncompetitive inhibitor for xanthine oxidase, improves the effects of chloramine and hydrogen peroxide, and promotes their toxic effects on micro-organisms (22). Since neopterin induces the effects of some potent reactive molecules, including H_2O_2, HOCl, and peroxynitrites, it is also known as an "endogen regulatory molecule organizing the cytotoxic effects of activated macrophages" (7). In our study we did not add any fresh media into the culture flasks and cells were not passaged further. The neopterin increase, especially after the first week, suggests that it may be due to the progressive oxidative stress in the cell culture.

In a study with acute IM, chronic IM, and XLPD patients Schuster et al. showed high neopterin and IFN-γ levels in acute infection were due to T lymphocyte activation while the B lymphocyte population infected via EBV grew (23). Although IL-6 levels were high in the patients with acute infection, it decreased in the chronic cases, pointing to the elimination of immortal B lymphocytes via cellular immune response. In the same study it was shown that high neopterin levels in acute infections were associated with the indirect effects of IFN-γ on neopterin, and not chronic transformation. Another explanation, given in the same hypothesis, was the high neopterin levels accompanying low IL-6 levels in XLPD patients. In our study IL-6 levels were still elevated at the third week of infection, which suggested that 3 weeks was not long enough to represent the chronic infection, and that the oxidative stress mechanisms in the cell culture were sufficient.

In recent years it has been shown that EBV may also infect T lymphocytes and NK cells in vitro. Although T lymphocytes constitute the main source of IFN-α and IFN-β production during many infections, they only secrete IFN-α after EBV infection (24). T cells may express variable amounts of CD21 but the mechanism of T cell transformation via EBV is still unknown. It has previously been shown that B cell transformation is more effective if T lymphocytes are depleted with cyclosporin A (15), but in our study we did not apply such a process because our aim was to measure other cytokine responses to EBV infection. IFN-γ levels were higher in cultures stimulated with PHA, a specific T cell mitogen, compared to the group stimulated with the B cell mitogen LPS (Figure 5).

In a previous study, Whittingham et al. (17) showed that TNF-α levels did not increase in either induced nor noninduced cell cultures, and they suggested that EBV may have an inhibitory effect on TNF-α production. In another study (25), TNF-α levels were measured at 48 h after transformation via EBV and found to be undetectable, and so researchers suggested that EBV has an inhibitory effect on TNF-α production at the gene transcription level. In our study, TNF-α started to increase after 72 h, and it reached its maximum level in week 2 (Figure 4), but there was no significant difference between the mean levels measured in weeks 1, 2, and 3. Jochems et al. had previously

reported that different cell lines could show different types of cytokine responses during an infection in vitro (26). The cell line in our study (B95-8 lymphoblastoid) was different from the other cell line (QIMR-WIL lymphoblastoid) used in the 2 other studies referred to previously (16,25).

Supporting the data that B lymphocytes transformed via EBV secrete IL-6 as a growth factor in cell culture (3), IL-6 levels increased after 12 h in our study too (Figure 6).

It is known that IFN-γ, TNF-α, and IL-6 may increase as a response to oxidative stress in vitro as well as in vivo. The decrease in both TNF-α and IFN-γ levels could be due to the noncontinuous stimuli, or to accumulating dead cells, or to the short life time of cytokines because of their protein structure.

In conclusion, in our study neopterin levels increased independent of both specific and nonspecific stimulators, and also of transformation. This increase was due to the oxidative stress, because growing cells in culture might have insufficient medium and milieu. Since the neopterin level increases nonspecifically, measuring it may be useful during acute viral infection, but not as a specific marker showing the viral transformation.

Acknowledgements

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