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Neopterin Production in SCID Mice Injected with Human Peripheral Blood Mononuclear Cells

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Abstract

Intraperitoneal transfer of peripheral blood mononuclear cells (PBMC) from human EBV$^+$ donors into severe combined immunodeficiency (SCID) mice is a suitable model for studying some aspects of lymphomagenesis and immune activation. Neopterin is a soluble immune marker which was found to be a useful indicator for immune activation processes in humans, e.g. to monitor immunological complications in allograft recipients or to predict prognosis in HIV-infected individuals. In contrast, this pteridine compound is normally synthesized in murine organism in only very low amounts. The measurement of neopterin concentrations in serum and urine should be feasible in SCID mice reconstituted with human PBMC. In this study, we examined the usability of this experimental model for monitoring human T cell activation by neopterin measurements. The production of neopterin by SCID mice after injection of freshly isolated human PBMC, purified B or T cells and cultured Epstein-Barr virus (EBV)$^+$ lymphoblastoid cells (LCL) was determined. It was found that neopterin can be detected early after injecting SCID mice with PBMC, whereas injection of purified human T or B cells did not result in neopterin production. Highest neopterin levels were detected in mice treated with LCL cells when developing lymphoma. We discuss the possible sources of neopterin along this process and its usefulness in this model.

Introduction

Human monocytes/macrophages produce and release large amounts of neopterin (6-D-erythro-trihydroxypropylpterin) upon stimulation with interferon-γ (IFN-γ) (1). Neopterin is synthesized from guanosine triphosphate (GTP) in the first step catalyzed by the GTP-cyclohydrolase I (EC 3.5.4.16) (2). Significant associations between enhanced neopterin formation and IFN-γ production were observed in various diseases (3), and the monitoring of neopterin concentrations has turned out to be a useful marker for activation of cellular immunity (4). In humans, increased neopterin concentrations in serum and urine are detectable in viral infections including human immunodeficiency virus type-1 (HIV-1) infection, various malignant disorders, autoimmune diseases and

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during allograft rejection episodes (4–8). Particularly high neopterin concentrations were observed in patients suffering form leukaemia and lymphoma.

The severe combined immunodeficiency (SCID) mouse, intraperitoneally (i.p.) injected with human PBMC was found to be a valuable tool to study lymphocyte function (9, 10). When PBMC from Epstein-Barr-virus (EBV)$^+$ donors are injected, most animals develop EBV$^+$ B cell tumors of human origin (11–13) closely recalling the immunoblastic opportunistic lymphomas frequently observed in immunocompromised patients (14, 15). The SCID mice model is therefore useful also to study some aspects of the lymphomagenesis process in humans. Neopterin is produced in relevant concentrations uniquely in humans and primates, but this is not the case in mice (16). Therefore its determination in serum and/or urine of human PBMC-SCID transferred (hu-PBMC-SCID) mice could provide an "*in vivo*" model for monitoring immune activation state of T cells without interference of the murine immune system. The aim of the present study was: 1) to investigate whether pteridines of human origin can be detected in the hu-PBMC-SCID mouse 2) to clarify the possible source of pteridines in this model 3) to evaluate whether pteridine concentrations in mouse urine can be employed as a marker of immune activation or B cell lymphoma development.

Materials and Methods

Cell source and preparation

PBMC were obtained from healthy EBV$^+$ volunteers undergoing lymphapheresis after their informed consent. EBV infection was determined by the presence of antiviral capsid antigen and anti-EBV nuclear antigen IgG. Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation as described (17). After isolation, the cells were washed in RMPI-1640 and counted. For T and B cell purification, monocytes were depleted from PBMC by plastic adherence. T and B cells were obtained by double-rosetting of monocyte-depleted PBMC with neuraminidase-treated (Sigma, St. Louis, MO, USA) sheep red blood cells as described elsewhere (18). Purity of isolated cells was assayed by cytofluorographic analysis. Enriched B cell populations contained 60–80% CD19$^+$ cells and <1% CD2$^+$ cells. Purified T cell populations were found to contain <1% CD19$^+$ cells.

Injection of mice

BALB/c and SCID mice (BALB/c backbone) were purchased from IFFA Credo (L'Abreisle, France) and they were maintained in our facilities under pathogen-free conditions. Prophylaxis against *Pneumocystis carinii* infection was administered as reported (18). All mice were screened for the "leaky" phenotype; animals showing >50 μg/ml IgG in serum were excluded from this study. To study neopterin production, mice of both sexes (ages 7–9 weeks) were inoculated i.p. with $7–10\times10^7$ unfractioned PBMC, $3–7\times10^7$ purified T or B lymphocytes, or with 1×10^6 cells of an established lymphoblastoid cell line (LCL) derived *in vitro* as reported elsewhere (19). Influence of cytokines on neopterin production was assessed by inoculating PBMC-injected mice i.p. with 100 μg anti-IL-2 or anti-IL-6 antibodies every 3 days for 30 days. Anti-IL-2 antibody from rabbit was kindly provided by Immunex (Seattle, WA, USA), goat anti-IL-2 and anti-IL-6 antibodies were purchased from Boehringer Mannheim (Mannheim, Germany); an additional anti-IL-6 antibody from goat was purchased from Genzyme (Boston, MA). In one set of experiments, B cell-injected mice were inoculated i.p. with 0.8 ml of supernatant from the EBV-producing B95.8 cell line, 2 days after cell transfer. Animals were observed every other day for signs of illness; when they became sick, they were killed by excess ethyl ether anaesthesia and autopsied. In every case, follow-up was completed after 36 weeks.

Sample collection and pteridine measurement

Urine samples were collected sterilly, and stored at −20 °C in the dark until testing. For some experiments urine samples from individual animals of the same experimental group were pooled before freezing. Blood samples were collected from the retro-orbital plexus, and serum separated by centrifugation was stored at −20 °C.

Urinary neopterin was assayed by high performance liquid chromatography (HPLC) as described previously (20). Positive results of HPLC measurements were confirmed by RIA test. Due to small volumes of specimens, urine was diluted 1 : 11 in potassium phosphate buffer (0.015 M, pH = 6.4), e.g., 20 µl urine plus 200 µl buffer. Then 10 µl of the specimen were injected into a HPLC system (LC 5500, Varian, Palo Alto, CA, USA) and separated on a C18 reversed phase column (LiChroSorb, Merck, Darmstadt, Germany). Neopterin was quantified by its native fluorescence (353 nm excitation, 438 nm emission wavelength at a signal noise ratio of 4). The lowest limit of detection in HPLC-measurements was 0.1 µmol/L. Thus, after dilution of urine specimens minimum detectable concentrations of neopterin were 1.0 µmol/L. For the second experiments, we were able to modify the dilution step to increase the sensitivity to 0.4 µmol/L.

Neopterin concentrations in serum samples were determined by radioimmunoassay (RIA, Henning, Berlin, Germany). Due to small sample volumes the detection limit of the test was 3.5 nmol/L.

Results

Neopterin concentrations in the urine of most untreated SCID and BALB/c mice were found to be below the test limit (< 1 µmol/L). Only two samples obtained from control untreated SCID mice contained neopterin concentrations above this level (1.0 and 2.0 µmol/L, respectively; Fig. 1). Injection of SCID mice with human PBMC was followed by a significant increase in neopterin production within the next days. Average neopterin concentrations in urine measured 9–68 days after injection was 2.10 ± 0.43 µmol/L (n = 13), and only two samples contained neopterin below the detection limit of

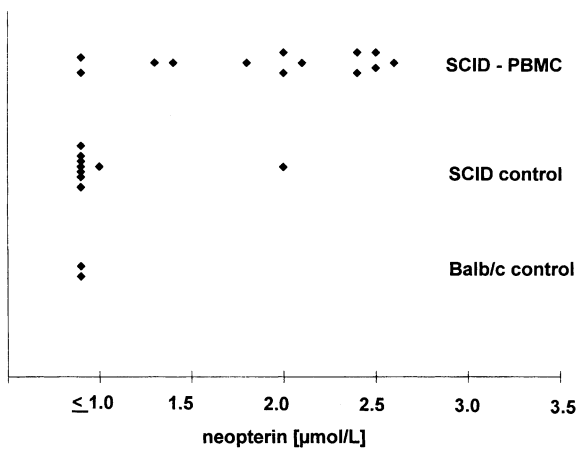

Fig. 1. Urinary neopterin levels in mice injected with human PBMC. SCID mice were inoculated i.p. with 7 to 10×10^7 unfractioned human PBMC. Neopterin concentrations were assessed 9–64 days after injection (limit of detection for diluted samples was 1 µmol/L neopterin). As controls urine was obtained from untreated SCID or BALB/c mice.

Table 1. Urinary neopterin concentrations (μmol/L) in PBMC-injected SCID mice.

	10 days	20 days	30 days	40 days
T cells	n. d.	1.1	n. d.	n. d.
B cells	n. d.	n. d.	n. d.	n. d.
B cells sn B95.8	n. d.	n. d.	n. d.	n. d.
PBMC	n. d.	2.3*	1.9*	4.5*
PBMC anti-IL-6	n. d.	1.0	n. d.	n. d.
PBMC anti-IL-2	2.0*	2.8*	n. d.	1.2

Mice were inoculated with $3–7\times10^7$ human T or B cells, or with $7–10\times10^7$ unfractioned PBMC. One group of mice was coinjected with B cells and the supernatant (sn) from the EBV-producing B95.8 cell line. PBMC-injected animals were cotreated i.p. with 100 μg anti-cytokine antibodies (anti-IL-6 or anti-IL-2) every 3 days for 30 days. Samples of 3–6 animals were pooled after collection (n = 3–6); n. d. = below the test limit of 1.0 μmol/L neopterin (* $p < 0.01$ compared to untreated SCID mice).

the test (Fig. 1). Concentrations of the pteridine in urine were found to be higher during the first 30 days after injection (range 1.3–2.6 μmol/L; n = 9) in this series.

To evaluate the cell population responsible for neopterin production in SCID mice, animals were injected with either $3–7\times10^7$ enriched human T or B cells, or with $7–10\times10^7$ unfractioned PBMC. Purified B cells did not cause any neopterin production in SCID mice measured 10–40 days after injection (Table 1). Accordingly, neopterin was not detectable in supernatants of the EBV-transformed B95.8 nor of *in vitro* established LCL cells (all concentrations < 2.0 nmol/L). When injecting mice with purified T cells, neopterin was detected at moderate concentrations 20 days after injection (mean 1.1 μmol/L). Treatment of mice with unfractioned PBMC resulted in an increase in neopterin production as early as 20 days following injection. On the other hand, treatment of PBMC-injected mice with two different anti-IL-6 antibodies markedly decreased neopterin formation, and borderline concentrations of neopterin were detected in a single peak 20 days after PBMC transfer (mean 1.0 μmol/L). In contrast, when mice were injected with PBMC and two different anti-IL-2 antibodies, neopterin production was detected earlier (2.0 μmol/L measured 10 days after injection) compared to animals injected with PBMC alone; but still neopterin formation in this group peaked 20 days following injection (2.8 μmol/L).

We next examined the behavior of neopterin concentrations in mice during the development of lymphoma in LCL injected SCID mice. In our experiments 12 out of 29 injected SCID mice developed lymphoma during 100 days of observation. Low neopterin concentrations in urine determined by ELISA test were detected in 9 out of 17 animals not developing lymphomas ranging from 0.40–0.59 μmol/L (Table 2). Three mice out of this group died before end of the study. There was no obvious time relationship detectable of neopterin production in these animals. Instead, among the group of LCL-injected mice which developed lymphomas, neopterin was detected in every animal, concentrations ranging from 0.40–14.75 μmol/L. Mean survival in this group was 55 days, no animal lived longer than 70 days after being injected with LCL and developing lymphomas. Ten days before developing lymphoma 11 out of the 12 SCID mice showed increased neopterin levels (7.41 ± 3.99 μmol/L), 20 days before tumor development

Table 2. Neopterin concentrations in SCID mice injected with LCL.

	10 d	20 d	30 d	40 d	50 d	60 d	70 d	80 d	90 d	100 d
without lymphoma										
neopterin detected	0/17	5/16	1/15	0/15	2/15	1/15	0/15	0/14	2/14	1/14
neopterin (μmol/L)	–	0.4±0.03	0.5	–	0.4±0.0	0.4	–	–	0.5±0	0.5
with lymphoma										
neopterin detected	2/12	0/12	6/12	4/11	7/9	6/6	2/2	–	–	–
neopterin (μmol/L)	0.8±0.4	–	0.6±0.2	0.6±0.2	3.6±3.6	7.9±3.6	10.6±4.1	–	–	–

SCID mice were injected with LCL and urinary neopterin concentrations were assessed by ELISA. During 100 days of monitoring 12 out of 29 animals developed lymphoma. The table lists the number of mice, the amount of samples with detectable neopterin levels (≥0.40 μmol/L), and the concentration values measured in the positive specimens (mean ± standard deviation).

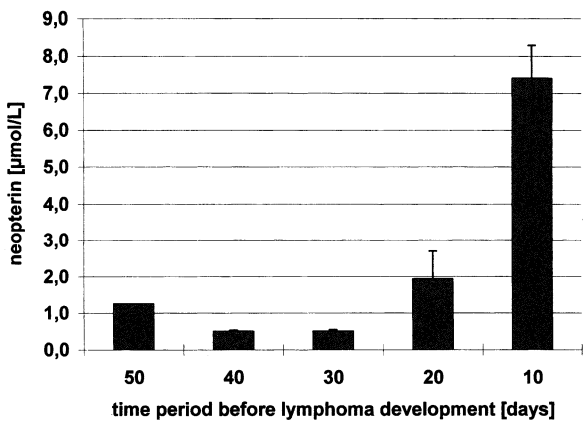

Fig. 2. Urinary neopterin concentrations (mean ± standard deviation) in LCL injected SCID mice developing lymphoma. Number of mice with detectable neopterin levels (limit of detection for diluted samples was 0.4 μmol/L): 1/9 (50 days before developing lymphoma); 3/11 (40 days); 4/12 (30 days); 8/12 (20 days); and 11/12 (10 days).

neopterin was detected in 8 animals (1.93 ± 3.30 μmol/L), 30 days before in 4 (0.50 ± 0.09 μmol/L), 40 days before in 3 (0.50 ± 0.06 μmol/L) and 50 days before lymphoma were diagnosed in 1 (1.25 μmol/L) mouse injected with LCL cells (Figure 2).

In the same group of animals, neopterin levels in serum were monitored by RIA test. Detectable serum neopterin concentrations were observed in 8 out of 12 animals developing lymphomas (mean 5.5 ± 2.9 nmol/L), in contrast to 3 out of 17 animals not developing lymphomas (mean 3.6 ± 0.6 nmol/L).

Discussion

Interperitoneal transfer of PBMC from EBV$^+$ donors into SCID mice leads to high human immunoglobulin levels in mouse serum and B cell lymphoproliferative disease. We previously demonstrated that these events depend on activation of the coinjected human T cells (18). In our study, production of the human pteridine neopterin was only detected in a small fraction of untreated SCID mice. This finding corresponds with earlier studies showing no detectable neopterin formation in normal mice (16). Significant neopterin levels could be detected early after transfer of unfractionated human PBMC into SCID mice, and these values were markedly increasing thereafter in these animals. In the SCID model, the human immune cells are functional and immune responses can be induced during the first 2–3 weeks. As transferred T cells become activated rapidly against murine antigens after PBMC injection (18), the early appearance of detectable neopterin concentrations in urine might be reasonably attributed to the presence of functional human monocytes/macrophages activated by T cells. This effect may be mediated by IFN-γ produced by transfected T cells, since previous data showed that IFN-γ producing TH1 cells undergo preferential activation in SCID mice (21).

Treatment of PBMC-injected mice with anti-IL-2 antiserum only partially affected the production of neopterin. This is in line with the present understanding that IFN-γ is the major stimulus of neopterin synthesis in monocytes/macrophages (1, 22). The finding that mice injected with purified B cells, or with purified T cells did not produce neopterin corresponds to the finding that monocytes/macrophages are the major source of the pteridine (1, 22), and it corresponds well with our result that neopterin was not detectable in mice injected with supernatants of the EBV-transformed B95.8 cells. Although neopterin production by B cells has been described under stimulation with IFN-γ or IL-2 (23), the presence of functional T cells within the inoculum seems to be required both for production of these cytokines and for B cell expansion (18). Therefore, with the data from our study, we can not exclude that B cells are a potential source of neopterin in the presence of activated T cells. This suggestion would be supported by the finding that anti-IL-6 treatment of mice transferred with unfractioned PBMC decrease neopterin formation, as IL-6 is a potent inducer of B cell proliferation. This conclusion is also supported by the association between increasing neopterin concentrations in mice developing lymphomas after LCL injection. In mice injected with LCL cells, which did not develop lymphomas, neopterin levels were relatively low, and decreased after 3–4 weeks, whereas very high neopterin concentrations were found only in animals at lymphoma presentation. The period in which lymphoma appear in hu-PMBL-SCID mice varies between 11–15 weeks (24); at this time only a few activated memory T cells and scarce B cells remain in the mice, and no human macrophages or other accessory cells are detectable (25). Then, macrophages appear to be unlikely to be the source of neopterin at the time of presentation of lymphoma. It remains unclear why high neopterin concentrations were present in mice developing lymphoma, whereas no neopterin could be detected in the *in vitro* preparation of the transformed cells. We hypothesize that modified lymphoma-B cells could be responsible which may depend on a certain biological environment. On the other hand, tumor generation in the hu-PMBC-SCID mice is associated with high serum levels of human immunoglobulins (24) indicating B cell proliferation and increased secretion. Thus, the high neopterin concentrations in urine early found in hu-PMBL-SCID mice could be sustained by a polyclonal activation and proliferation of B

cells. Probably, the early appearance of neopterin concentrations reflects activation of T cells, which in turn activate monocytes to produce the pteridine.

In conclusion, the SCID mice produce detectable amounts of neopterin only when injected with unfractioned PBMC from human EBV$^+$ donors. Neither inoculation with purified human T cells or B cells has a comparable effect. Animals in which lymphoma develop show a further increase in neopterin production preceding the onset of tumor development. Neoplastic B cells could be responsible for this increase in neopterin synthesis. In this model, neopterin concentration in murine urine allows easy monitoring of early lymphoma development without necessary bleeding of the animal. In the hu-PBMC-SCID mice model – although neopterin is produced while the transferred cells function in a regulated activated immune system – quantification of neopterin concentrations does not seem useful for monitoring residual T cell activation at time of tumor development when probably tumoral B cells are protagonists.

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