Pteridines

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Abstracts

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Synthesis and Properties of 7-Hydroxylamino-1,3,6-trimethylumazines

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Tautomerism plays an important role in organic and especially in nitrogenheterocyclic chemistry. Base-pairing in DNA is due to hydrogen bonding between amide and amino functions showing that hydroxy groups adjacent to ring-N-atoms adapt the lactam configuration whereas an amino group does not tautomerize to an imino-dihydro structure.

Reinvestigation of the alleged 4-hydroxylamino-1,3-dimethyluracil (1) structure by ¹H-NMR spectra revealed that the preferred tautomeric form is presented by the oxime structure (2) in analogy to the trioxohexahydro form of barbituric acid. Structure 2 was furthermore proven by comparison of the UV spectra of 2 with the tautomerically fixed 1,3,5,5-tetramethyl- barbituric acid monoxime (3) and 1,3-dimethyl-6-N-methyldroxyaminouracil (4). The UV spectra of 2 and 3 are very similar and absorb at about 250 nm whereas 4 is bathochromically shifted to 277 nm.

Extension of these studies to the 7-hydroxylamino-1,3,6-trimethylumazine series, obtained from

7-chloro-1,3,6-trimethylumazine (5) and hydroxylamine and its derivatives, indicated that four tautomeric forms 6-9 have to be considered.

Synthesis of the model substances 6-O-ethylhydroxylamino- (10), N-methyl-hydroxylamino- (11) and N,O-dimethyloxylamino-1,3,6-trimethylumazine (12), determination of their pK-values and comparisons of the UV-spectra of the neutral and monoanion species showed unambiguously a vinylous hydroxamic acid structure of all of these compounds. The shift of the equilibrium towards this tautomeric form (6) seems to be due to a strong resonance interaction between the hydroxylamino and the carbonyl group in 4 position of the lumazine nucleus.

Pfeleiderer W, Ferch H : Liebigs Ann Chem 1958; 615 : 52

Urinary Neopterin Levels in Familial Mediterranean Fever

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Familial Mediterranean Fever (FMF: also known as recurrent hereditary polyserositis) a chronic inherited illness of obscure aetiology is a disease characterised by recurrent attacks of abdominal, pleuritic pain by fever. There is no specific diagnostic test to monitor treatment of FMF. Colchicine is used in order to prevent the attacks. Patients with FMF have deficient suppressor cell function via a prostaglandin-mediated mechanism.

In the present study, it has been aimed to define a possible relationship between neopterin and FMF disease. Therefore we have studied 72 patients with FMF (37 male, 35 female, mean age 28.12 ± 8.43 years). 34 of them were in remission and 38 had an acute attack. We found that mean urinary neopterin concentration in FMF patients with attack was $228.37 \pm 137.94 \mu\text{mol}$ neopterin/mol creatinine whereas the concentrations of neopterin in remission and the control group were 146.53 ± 93.74 and $136.94 \pm 57.66 \mu\text{mol}$ neopterin/mol creatinine, respectively. There was a statistically significant difference between the group of patients with acute attacks and the group in remission and the healthy controls ($p < 0.05$).

In this study, urinary neopterin concentrations in the groups treated with colchicine have been found lower than others. It seems that colchicine therapy may cause a decrease of neopterin levels. Our study shows that there may be an association between cell-mediated immunity and FMF disease.

Elevated Neopterin Levels and Reduced Dihydropteridine Reductase in Neonates; a Test for Early Neurological Disease ?

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5,6,7,8-Tetrahydrobiopterin is a cofactor for certain monooxygenases of amino acids. The biosynthesis of 5,6,7,8-tetrahydrobiopterin starts with the conversion of guanosine-triphosphate to form 7,8-dihydroneopterin-triphosphate as an intermediate

by GTP-cyclohydrolase I, which is then further converted to form 5,6,7,8-tetrahydrobiopterin by pyruvoyl-tetrahydropterin synthase (PTPS). Finally, the cofactor is recycled by dihydropteridine reductase. Due to low activity of PTPS human macrophages are unique producing and releasing excessive amounts of neopterin upon stimulation with interferon- γ , and the accumulation of 5,6,7,8-tetrahydro-biopterin is very low. Increased neopterin concentrations are found in patients suffering from diseases known to be associated with immune activation such as infections, autoimmune disorders and certain types of cancer. More recently neopterin concentrations were reported to be also increased in serum, urine and/or cerebrospinal fluid of patients with neurodegenerative diseases such as Alzheimer's, Huntington's and Parkinson's disease and in children with autism. At the same time in these patients a reduced activity of dihydropteridine reductase is frequently seen. Likewise, urinary neopterin was significantly raised in twelve Rett's children between birth and four years old. Again, high neopterin levels have been found to be associated with low activity of dihydropteridine-reductase. During the later course of the disease, neopterin concentrations returned to normal. The data indicate that immunological abnormalities may be involved in the pathogenesis of Rett's syndrome which is important in the early phase of the disease. The coexistence of elevated neopterin levels and reduced dihydropteridine reductase appears to be indicative for a variety of neurological diseases. Therefore, screening for elevated neopterin levels and reduced dihydropteridine reductase in neonates might serve as a test for early neurological disorders.

Is Dihydropteridine Reductase a Negative Acute Phase Protein ?

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The human organism responds to tissue damage due to injury, trauma or tissue infection by implementing the acute phase response. The cell most strongly involved in initiating the acute phase response is the tissue macrophage or blood monocyte. Bacterial products can additionally activate monocytes and macrophages stimulating the acute phase

response cascade which involves a broad spectrum of mediators and cytokines including interleukin-1 and tumour necrosis factor- α . Usually the acute phase response returns to normal within 48 hours, however, this normal pathway can be extended by the persistence of stimulation.

Low activity of dihydropteridine reductase was found in patients with neurodegenerative disorders like Alzheimer's, Huntington's and Parkinson's disease and in children with Rett's syndrome and autism. In the same groups of patients increased neopterin concentrations are frequently found which indicates activated cellular immune response. Cytokine cascades being involved in the cell-mediated immune response and especially the products of activated macrophages such as interleukin-1 and are well known to contribute and initiate inflammation. The occurrence of Inflammation is indicated by enhanced serum levels of, e.g. C-reactive protein and serum amyloid A, but also by the formation of cytokines such as interleukin-6. In addition, disturbances of iron and zinc metabolism take place leading to decreased serum zinc and iron levels. Both changes were found to correlate to inversely to the increase of neopterin in patients with infections and malignancy. It appears that the activity of dihydropteridine reductase is depressed during such an inflammatory response. The data imply that dihydropteridine reductase is a kind of a negative acute phase protein.

Evaluation of a Commercial ELISA for Serum Neopterin Measurement

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The evaluation of the ELITEST-HENNING for neopterin measurements had two aims. We examined the feasibility of this test for routine application in clinical chemistry: diagnosis, follow up and prognosis in certain malignant diseases, in autoimmune diseases, to provide differential diagnosis of inflammatory disorders and non-inflammatory conditions, to confirm diagnosis of viral infections and to ascertain allograft rejection in organ transplantation.

In addition, we investigated the application of this ELISA in transfusion medicine to guarantee more

safety in blood transfusion, to reduce the loss of donor blood based on false-positive neopterin levels by possible preanalytical or analytical failures and to examine the influence of slightly reduced renal function on neopterin concentrations in serum of blood donors. Elevated neopterin concentrations in donor blood (>10 nmol/l) lead to the exclusion of this blood from transfusion. Therefore 2-3% of donated blood are generally eliminated.

Our data show that even very high concentrations of haemoglobin in serum (>100 mg/dl) have no influence on neopterin values in this test. As well, hypertriglyceridaemia (<700 mg/dl), hypercholesterolaemia (>300 mg/dl) and hyperbilirubinaemia (<4 mg/dl) have no effect on neopterin values. The accuracy of the ELISA is satisfactory and in accordance to the rules of clinical chemistry (intra-assay CV 3.8%, interassay CV 7.9%). Different incubation temperatures (25-37°C) did not alter the test results. The incubation time for all samples within one series has to be kept equal, shorter incubation periods leading to reduced formation of antigen-antibody complexes and therefore to higher neopterin values. No correlation was found between neopterin and creatinine concentrations in serum of 110 blood donors. Nevertheless 10% of elevated neopterin levels were normalized by calculation of the neopterin per creatinine ratio. However, it seems to be critical to release blood with elevated neopterin due to increased creatinine. The reason for renal impairment as most likely cause of elevated creatinine needs to be clarified.

We conclude that elevated neopterin concentrations in blood donations can almost exclusively be referred to enhanced endogenous formation of neopterin due to underlying immunopathologies and are not the result of analytical pitfalls.

Performance of Serum Neopterin Measurement in Blood Banks

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For the measurement of neopterin in large numbers of specimens radioimmunoassay techniques were introduced in the middle of the eighties and since around 2 years enzyme linked immunosorbent assays (ELISA) are commercially available especially for the screening of blood donations in blood banks.

In autumn 1986 we started with the screening for serum neopterin in blood donations by a radioimmunoassay (IMMUTest Neopterin / HENNINGtest Neopterin, Henning-Berlin), but in 1994 we changed to the first commercially available enzyme-linked immunosorbent assay (ELItest Neopterin-Screening, Henning-Berlin). This test had been evaluated by our institute in the years before. We could demonstrate: - the ELISA is better suitable for its application in the context of transfusion medicine where the primary goal is to differentiate between neopterin concentrations below versus above a cut-off limit of 10 nmol/l; compared with RIA the ELISA showed significantly less "false positive" results, no outliers and higher reproducibility.

Considering these results we adapted the test to an almost fully automatic system consisting of a diluter (Hamilton ATplus 2) and an analyzer (BE-PIII, Behring ELISA Processor III) for automatic incubation, washing, dispensing and photometric measurement. Measured results are calculated by the help of a point to point or cubic spline standard curve (in a study on 724 samples we could show that there is no statistically significant difference between the calculation of concentrations by cubic spline or point-to-point curves, especially in the most important range around 10 nmol/l). A plate is valid when the concentrations of the control sera are in the corresponding range. We recommend the determination of an internal quality control on each plate. This should be a sera pool with a concentration around 10 nmol/l. After this procedure the concentrations are documented. All samples with an initial concentration ≥ 9.5 nmol/l and < 16 nmol/l are repeated once, samples between 16 and 22 nmol/l twice. Samples with values ≥ 22 nmol/l are not determined anymore (the first value is the result). From all determinations the mean value is calculated and if this value is higher than 10 nmol/l the respective donation is withdrawn.

The performance of neopterin measurement does not show any big technical or logistical problems. Especially the ELISA is well-suited for screening of blood donations. Combined with a sophisticated and automatized equipment a high number of samples can be screened without interfering with the other required tests.

Apoptosis in HIV Infection : a Potential Role for Neopterin and 7,8-Dihydroneopterin

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Recent papers have proposed that apoptosis, a type of programmed cell death (PCD), is the cause of immune-cell loss in patients infected with human immunodeficiency virus. An excessive production of reactive oxygen species (ROS) and a broad deficiency of antioxidant mechanisms, both provide strong support for the premise that oxygen species (OS) exist in HIV infection and AIDS. Interferon- γ (IFN- γ) upregulates the expression of tumor necrosis factor receptors in a variety of cells and induces the secretion of neopterin and 7,8-dihydroneopterin in human macrophages.

We have investigated the effect of neopterin and 7,8-dihydroneopterin on the tumor necrosis factor- α (TNF- α) induced apoptosis of the monocyte/macrophage-like cell line U937 cells as determined by propidium iodide staining of isolated nuclei. Increasing concentrations of neopterin (up to 1 mM, saturated solution) and of 7,8-dihydroneopterin (up to 300 mM) slightly decreased TNF- α induced apoptosis. In contrast, a higher dose of 7,8-dihydroneopterin (5 mM) superinduced PCD mediated by TNF- α . In the absence of TNF, 7,8-dihydroneopterin (5 mM) did not cause apoptosis of U937 cells. The effect of 7,8-dihydroneopterin on TNF- α mediated PCD was downregulated by the addition of antioxidants N-acetylcysteine (NAC) and superoxid dismutase (SOD). The role of reactive oxygen intermediates was studied in two additional test systems. Neopterin (up to 1 mM) and 7,8-dihydroneopterin (up to 1 mM) scavenged, whereas the high dose of 7,8-dihydroneopterin (5 mM) enhanced hydrogen peroxide-induced luminol-dependent chemiluminescence. As determined in a cytofluor plate reader, reactive oxygen intermediates are generated in U937 cells when they are incubated with a combination of 5 mM 7,8-dihydroneopterin and TNF- α . In conclusion, our data demonstrate, that neopterin derivatives interfere with PCD induced by TNF- α . While neopterin and low doses of 7,8-dihydroneopterin may decrease cell death, high concentrations of 7,8-dihydroneopterin in combination with TNF- α lead to formation of unphysiologically high levels of reactive oxygen intermediates superinducing apoptosis most likely due to oxidation stress on cells. Based on these findings it is therefore conceivable that pteridines, in particular neopterin derivatives released by activated macrophages, directly

interfere with the cells ability to maintain an appropriate oxidant/antioxidant balance and may well promote the processes of PCD and latent virus activation in HIV infection.

The Differential Impact of Interferon- γ on Pro- and Antiinflammatory Mediator Synthesis during Homeostasis versus Traumatic Stress

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Exogenous substitution of IFN- γ , a key mediator for adequate forward-regulatory monocyte immunoperformance, has been advocated to overcome post-traumatic mononuclear leukocyte paralysis. Conversely IFN- γ also appears as a potent proinflammatory mediator, thus contributing in sepsis to capillary leakage-driven organ failure. It was the objective of this investigation to further define the potential of IFN- γ as a modifier of monocyte activity under pre-and post-traumatic conditions. To study this, EDTA blood from 19 patients (12 male, 7 female; 685 years) undergoing cardiac surgery under extracorporeal circulation was incubated (37°C 6% CO₂) under continuous rotation pre-operatively (D-1), 6h post-operatively (D0) as well as on post-operative days (D) 1, 2, 3 and 5 with LPS (1 g/ml) for 12h in the presence or absence of human recombinant IFN- γ (10 ng/ml). Following termination of the blood cultures the plasma levels for the fo-

llowing pro-and anti-inflammatory cytokines were determined via ELISA and bioassay, respectively: TNF- α (WEHI 164*), soluble TNF receptors (sTNFR-75), IL-1, IL-1 receptor antagonist (IL-1Ra) and IL-6 (7TD1*). Furtheron the monocyte metabolite neopterin as a marker of cellular activity as well as immunoreactive prostaglandin-E, a crucial endogenous downregulator of T-cell IL-2- as well as of monocytic TNF-activity, were determined via ELISA.

LPS induced release of TNF- α and IL-6 was clearly augmented under IFN- γ substitution with - most strikingly - the degree of post-operative TNF- α enhancement achieved, being significantly ($p < 0.01$) higher (3-12 fold) compared to the preoperative enhancement. While IFN- γ could also rather consistently enhance the post-operative IL-1Ra release, as well as prostaglandin-E synthesis, there was no effect seen for IL-1 and sTNFR release. Surprisingly, neither LPS nor LPS + IFN- γ could further enhance the post-operative elevated levels of neopterin in untreated cultures. Thus, there is a wide spectrum of differential IFN- γ activity on monocyte activation including also anti-inflammatory properties. As apparently cellular preactivation facilitates the monocyte reactivity towards IFN- γ , we conclude that its exogenous administration should be effective in states of trauma-induced immunoparalysis but must be carried out with great caution in states of profound inflammation.

Tumor Necrosis Factor- α , Interferon- γ and Neopterin Production in Vivo

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Interactions between cytokines are considered to play an important role in the pathogenesis of a variety of disorders. Like a two-edged sword, they contribute to immunosurveillance as part of the host's immune response but may also be involved in the precipitation of specific symptoms. This is particularly true in HIV infection when cytokines on the one hand may limit the spread of virus by enhancing specific immune response and, on the other hand, increasing the reproduction rate of virus

	TNF- α (U/ml)	IL-1 β (ng/ml)	IL-1Ra (pg/ml)	IL-6 (U/ml)
pre-op. LPS	24 $\pm$ 6	33 $\pm$ 22	22391 $\pm$ 5807	124 $\pm$ 25
D-1 + IFN- γ	38 $\pm$ 7	32 $\pm$ 21	25905 $\pm$ 5146	281 $\pm$ 105*
D-0 LPS	4 $\pm$ 1*	47 $\pm$ 31	52352 $\pm$ 11956*	157 $\pm$ 31
+ IFN- γ	46 $\pm$ 14*	67 $\pm$ 45	95986 $\pm$ 19777*	383 $\pm$ 83*
D-1 LPS	8 $\pm$ 1*	24 $\pm$ 16	83391 $\pm$ 1826*	150 $\pm$ 27
+ IFN- γ	61 $\pm$ 18*	27 $\pm$ 18	90824 $\pm$ 16249	412 $\pm$ 71*
D-2 LPS	19 $\pm$ 8	57 $\pm$ 39	31311 $\pm$ 34501*	104 $\pm$ 18
+ IFN- γ	77 $\pm$ 19*	55 $\pm$ 35	84441 $\pm$ 23533*	347 $\pm$ 64*
D-3 LPS	34 $\pm$ 16	32 $\pm$ 21	33505 $\pm$ 8757	129 $\pm$ 20
+ IFN- γ	77 $\pm$ 25*	38 $\pm$ 25	56688 $\pm$ 17082*	462 $\pm$ 72*
D-5 LPS	18 $\pm$ 4	55 $\pm$ 36	33321 $\pm$ 5595	122 $\pm$ 33
+ IFN- γ	69 $\pm$ 24*	55 $\pm$ 37	51295 $\pm$ 7727*	321 $\pm$ 93*

*Mean $\pm$ SEM; * $p < 0.05$ pre- versus post-op.; * $p < 0.05$ LPS + IFN- γ versus LPS)

by probably inducing replication.

IFN- γ is the main inducer of neopterin formation by human monocytes/macrophages. Enhanced concentrations of both markers indicate activation of cellular, so-called TH1-type, immune response. TNF- α is able to enhance formation of neopterin from IFN- γ stimulated monocytes/macrophages in vitro in a synergistic manner. In a variety of diseases a correlation between serum concentrations of IFN- γ and neopterin was observed. However, there is no clear information about the influence that TNF- α may have on the formation of increased neopterin concentrations in patients. We determined serum concentrations of TNF- α (ELISA, Medgenix, Brussels, Belgium), IFN- γ (RIA, Centocor, Malvern, PA) and type 75 kD soluble receptor for TNF- α (ELISA, Hoffman La Roche, Basel, Switzerland) and of urinary neopterin (HPLC as described) in 63 patients with HIV infection. In a significant proportion of patients increased concentrations of the analytes were found: 22/63 patients (35%) had detectable TNF- α , 55/63 (87%) had increased (above 4.8 ng/mL) soluble TNF receptor; 49/61 patients (80%) had increased serum IFN- γ , and urinary neopterin was increased in 60/63 (95%). Correlations of soluble TNF-receptors with TNF- α ($r_s=0.45$, $p<0.001$), neopterin ($r_s=0.52$, $p<0.0001$) and IFN- γ ($r_s=0.51$, $p<0.0001$) were found. There existed no such association between TNF- α and neopterin concentrations ($r_s=0.22$, $p>0.8$). Similar data were obtained in patients with autoimmune disorders, e.g., systemic lupus erythematosus, and in patients with malignant diseases. In all these clinical situations, a strong correlation between neopterin concentrations and sTNF-receptors was evident.

Our data indicate that IFN- γ , more directly than TNF- α , is involved to trigger neopterin formation in vivo. In fact, there was no correlation between the concentrations of TNF- α and neopterin in the patients with HIV infection. However, there appears to exist a more general interaction in various groups of patients between the TNF- α and IFN- γ system. TNF- α and IFN- γ seem to synergistically contribute to an upregulation of the immune response. The concomitant appearance of shed sTNF receptors may result from endogenous downregulation of responsiveness to TNF- α of cells stimulated with IFN- γ .

Neopterin Concentrations in Different Body Fluids

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During the past years the role of neopterin as marker for cell-mediated immunity in malignant diseases and HIV-infection was elucidated. However, the basis of neopterin production in various body fluids is not clear.

Neopterin concentrations in the urine and in the serum show an age and sex dependency. Mean urinary neopterin values in healthy women are ranging from 124-156 $\mu\text{mol/mol}$ creatinine. Mean neopterin concentrations in the serum of healthy subjects are ranging age dependently from 5.3 to 9.7 nmol/l. Cyst fluid obtained by laparoscopic puncture from benign ovarian cystadenomas had a median neopterin value of 4.8 nmol/l. In patients with advanced ovarian carcinomas ascites revealed a median neopterin concentration of 22 nmol/l. Furthermore, neopterin is present in the amniotic fluid. A median neopterin concentration of 15 nmol/l was measured during gestational week 16-34, and 48 nmol/l during week 35-40. Amniotic fluid obtained transvaginally from patients with premature rupture of membranes revealed a median neopterin value of 159 nmol/l. Neopterin is also present in vaginal secretions. In healthy women neopterin values are rather low (mean: 4.2 nmol/l). In women with vaginal infections neopterin concentrations were significantly higher (mean: 40.7 nmol/l; $p=0.0012$, Mann-Whitney U-test).

In conclusion, the presence of neopterin in various body fluids may derive from a local production. This would make neopterin a sensitive marker for the local enhancement of cell-mediated immunity.

Stereochemistry of Drosopterins

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The structures of droso- and isodrosopterins have been elucidated in 1978 showing a complex constitution with an unusual pentacyclic skeleton. So far, however the stereochemistry derived from the two chiral centers at position 6a and 6b could not be unraveled by normal physical methods. The question is whether the enantiomeric pair droso-/isodrosopterins is the cis (1) or the trans isomer (2) re-

garding the two central H-atoms. Unfortunately, no crystals could be obtained by various methods of crystallisation excluding an X-ray analysis as structural proof.

Another approach to solve the stereochemical problem was now based upon high-resolution ^1H -NMR spectra using a 600 MHz spectrometer. The spectra were determined in very dilute $\text{DCl}/\text{D}_2\text{O}$ from solubility reasons and revealed well separated signals of the various protons located at C-(6), C-(6a), C-(6b) and C-(7) in the range of 2.8-4.5 ppm. The signals were sharpened by a Lorentz-Gauss transformation allowing a very accurate determination of the coupling constants. The chemical shifts were assigned by the coupling interactions and 2D-COSY spectra. In addition an inverse detected ^1H - ^{13}C correlation (HMQC) revealed undoubtedly that H-C(6a) resonates at lowest field at 4.36 ppm and H-C(6b) at highest field at 2.79 ppm. Unfortunately, the coupling constant between H-C(6a) and H-C(6b) does not allow to solve the stereochemical relation due to some molecular disturbance leading to a $^3J_{\text{H-H}}$ of 8.59 Hz which accounts according to the Karpuls equation for the cis and trans orientation.

Finally the distances between the H-atoms have been determined by rotating frame NOE spectra indicating clearly that the H-C(6a) and H-C(6b) show the biggest distance of 320 pm which is in agreement with a trans arrangement of the H-atoms at the chiral centers.

Inter-proton Distances : Rotating frame NOEs in (pm) ROESY :

6a - 6b	320	6a - 7 β	240
6a - 6a	310	6b - 7 β	230
6a - 6 β	250	6b - 7 α	230
6a - 7 α	260		

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Attempts Towards the Synthesis of 6-(p-Azidobenzoyloxymethyl)-5,6,7,8-tetrahydropterin

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The biochemical conversion of L-phenylalanine into L-tyrosine is catalysed by L-phenylalanine-4-monooxygenase which is able to bind molecular oxygen and transfers hydroxyl groups to the phenyl ring of the substrate. Tetrahydrobiopterin is the natural cofactor of this reaction but more simple tetrahydropterins function analogously whereby a redox shuttle between the tetrahydro and the quinonoid dihydro state is operating. In order to get more information about the enzyme binding site for the cofactor the synthesis of a tetrahydropterin derivative bearing a photoreactive azidophenyl function was undertaken in order to label the enzyme by irradiation.

All attempts to synthesize 6-(p-azidophenyl)-5,6,7,8-tetrahydropterin have so far failed due to the incompatibility of the azido function with the tetrahydropterin state. Another photolabile cofactor was seen in the 6-(p-azidobenzoyloxymethyl)-5,6,7,8-tetrahydropterin (4) which should be derived from 6-hydroxymethylpterin (1). Hydrogenation of 1 led to 6-hydroxymethyl-5,6,7,8-tetrahydropterin which was stabilized by protection of H-N(5) by the p-nitrophenylethoxycarbonyl (NPEOC) group which can be cleaved by DBU in a β -elimination process under aprotic conditions without harming an ester function. Since the NPEOC group was applied for the first time for tetrahydropterin protection its stability was studied in model reactions.

Conversion of 6,7-dimethyl-N²-isobutyryl-5,6,7,8-tetrahydropterin with p-nitrophenylethoxycarbonyl chloride (NPEOC-Cl) into 6,7-dimethyl-N²-isobutyryl-N⁵-p-nitrophenylethoxycarbonyl-5,6,7,8-tetrahydropterin

dropterin worked with 40%. Treatment with methanolic ammonia cleaved only the isobutryl group and subsequent treatment with 0.5 M DBU in DMF afforded 6,7-dimethyl-5,6,7,8-tetrahydropterin which was isolated as its less soluble sulfated salt.

Reaction of 6-hydromethyl-5,6,7,8-tetrahydropterin with NPEOC-Cl gave selectively the N⁵-p-nitrophenylethoxycarbonyl derivative 2 which on treatment with p-azidobenzoic anhydride formed 6-(p-azidobenzoyloxymethyl)-5-(p-nitrophenyl-ethoxycarbonyl)-5,6,7,8-tetrahydropterin (3) in 30% yield. The removal of the NPEOC group in 3 and the isolation of the anticipated 6-(p-azidobenzoyloxymethyl)-5,6,7,8-tetrahydropterin (4) is under investigation.

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The Concentrations of Neopterin in Plasma, Ascites and Ovarian Cyst Fluid are Increased in Patients with Malignant Ovarian Tumours Compared with Benign Ovarian Tumours

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Ovarian cancer has the worst prognosis of all gynaecological malignancies. Furthermore, difficulties exist in discriminatory between malignant and benign persistent ovarian cysts, leading to a substantial number of women undergoing surgery that may not be necessary. A test that would aid in making this differential diagnosis would be of benefit. It is known that ovarian surface epithelium secrete bioactive cytokines. In patients with ovarian carcinoma increased serum levels of interleukin-6, interleukin-2 and neopterin have been found.

The aim of the present study was to determine if the concentration of neopterin differs in ascites and ovarian cyst fluid between patients with ovarian cancer and those with benign ovarian tumours.

The neopterin concentration was significantly higher in patients with ovarian cancer compared to benign ovarian tumours of the same size, in plasma 7 (median) nmol/l (7-9.8) (25-75 percentile) versus 5 (5-7), $p < 0.01$, in ascites 9.2 nmol/l (7.4-11.4) versus 5.7 (<2-6.1), $p < 0.01$ and in ovarian cyst fluid 11.3 nmol/l (7.6-19.2) versus 5 (3.1-7.2), $p < 0.001$.

The study suggests that activation of cell-mediated immunity is increased in patients with ovarian can-

cer compared to those with benign ovarian tumours. Neopterin determination might be a useful tool in the preoperative assessment of patients with persistent ovarian cystic tumours.

Does Neopterin Stimulate Inducible Nitric Oxide Synthase in Vascular Smooth Muscle Cells ?

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Neopterin was shown to decrease erythropoietin (EPO) mRNA expression and EPO protein formation in HepG2 cells in vitro. Recently, we found that the endogenous nitric oxide-donor sodium nitroprusside suppresses EPO mRNA expression, too. Therefore, we investigated whether neopterin derivatives, namely neopterin and 7,8-dihydroneopterin, modulate iNOS mRNA expression in vitro.

Cultured rat aortic vascular smooth muscle cells (VSMC) from female Wistar Kyoto rats were incubated with neopterin (200 μ M), lipopolysaccharide (LPS, 5 mg/ml), and interferon- γ (IFN- γ , 100 units/ml) for 9h. iNOS mRNA expression as measured by competitive reverse transcription polymerase chain reaction was stimulated by neopterin (1 amol/ μ l), LPS (2.5 amol/ μ l), and IFN- γ (10 amol/ml), but not by 7,8-dihydroneopterin. When cells were coincubated with neopterin and LPS, a more than additive effect on iNOS mRNA expression was observed (5 amol/ml). Coincubation of VSMC with 7,8-dihydroneopterin and LPS decreased the LPS induced stimulation of iNOS mRNA formation from 2.5 amol/ μ l to 1 amol/ μ l.

The present study demonstrates a neopterin-induced increase in iNOS mRNA expression on the transcriptional level in VSMC. While 7,8-dihydroneopterin has no direct effect on iNOS mRNA, it suppresses the LPS induced augmentation of iNOS mRNA expression. These data support the concept that pteridines per se exhibit biochemical functions, e.g. there might be an interaction between pteridines produced by macrophages and VSMC in clinical disorders associated with endothelial cell damage.

Uveitis and Different Systemical diseases Analysis of Serum-Parameters

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Patients with an underlying rheumatoid disease or sarkoidosis were compared with patients suffering from the same diseases but had additionally an intraocular inflammation. A further group were patients with uveitis but without systemical diseases. The question was if an additional intraocular inflammation leads to higher immune response and if only a local immune reaction in case of uveitis will be reflected as an increase of different serum parameters systemically. 67 patients were included into this study. Serum samples were taken before and at different time-points after therapy; neopterin, heat-shock protein (HSP), soluble IL-2-receptor (sIL-2R), soluble TNF-receptor (sTNF-R) and soluble CD23 were measured.

Elevated levels of neopterin ($p=0.002$) and soluble IL-2R ($p=0.0004$) were obtained from sarkoidosis patients with or without uveitis compared to normal controls. All concentrations of serum parameters of chronic forms of rheumatoid diseases were within normal ranges. Even if patients had additionally uveitis, no differences could be observed. In the patients' group with uveitis alone higher serum levels were obtained depending on the grade of inflammation.

From our observations we can conclude that a) sarkoidosis in combination with uveitis does not lead to higher serum levels than sarkoidosis alone, b) rheumatoid diseases with or without ocular inflammation are not reflected in higher concentrations of serum parameters and c) in special conditions of a local intraocular inflammation the immune response is detectable as higher serum values.

Influence of New Purine Analogues on Neopterin Formation in Human Monocytic Cells Stimulated with Interferon- γ

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The objective of this study was to determine the effect of various concentrations of three recently developed purine analogues, 2-Chlorodeoxyadenosine (2-CdA), Gemcitabine (dFdC) and Fludarabine (F-Ara-A) on the neopterin production by the human monocytic line THP-1, which behaves like freshly isolated human monocytes/macrophages in terms of pteridines biological activity.

CdA and F-Ara-A have been found to be effective in human lymphoid malignancies like CLL and NHL of low risk. CdA seems now to be the drug of choice for treatment of hairy cells leukemia. dFdC is a new analog of deoxycytidine which has demonstrated excellent antineoplastic activity against many kinds of solid tumors and leukemic cell lines. A common side effect of these drugs is immunosuppression which persists in treated patients for about 6 months.

For a toxic effect the purine analogs require the phosphorylation by deoxycytidine kinase to the triphosphate which incorporated into DNA causes cell death. In the MTT assay the effect of the different compounds on the growth inhibition of THP-1 cells was measured. The results show that THP-1 cells were completely inhibited by CdA, dFdC and F-Ara-A at a concentration of 10 nM, 8 nM and 400 nM, respectively, indicating high levels of deoxycytidine kinase in this cell line. When neopterin production by THP-1 cells in the presence of various concentrations of CdA, dFdC and F-Ara-A with or without Interferon- γ (IFN- γ) was measured, CdA and dFdC alone or in combination with IFN- γ did neither increase nor decrease the levels of neopterin at all concentrations tested. F-Ara-A, however, dose dependently induced an increased neopterin formation by THP-1 cells. In the presence of IFN- γ , the optimal trigger for neopterin production, F-Ara-A potentiated the levels of neopterin by three times compared to controls containing only IFN- γ . The relevance of the increased formation of neopterin by THP-1 cells treated by F-Ara-A for the clinical outcome in hematological malignancies has to be elucidated by measuring neopterin levels in vivo in patients treated by F-Ara-A.

Quality Testing of Immunodiagnostics in Austria

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At present, HIV and neopterin immunoassays are tested for their quality at the BSPI an released batch

by batch. Routine screening for HIV antibodies in single donor plasma and sera is done in blood banks and diagnostic laboratories, whereas neopterin is screened only in blood banks to improve the virus safety of blood and plasma donations. Both test *categories must be accurate, precise, re-producible* and consistent from lot to lot. For the evaluation of HIV antibody ELISA and Western blot tests, control sera used in our laboratory include dilutions of polyclonal pools and cocktails of human monoclonal antibodies to the transmembrane protein gp41 as well as seroconversion sera. Because of the high variability of the immune response to the HIV infection, sensitivity of antibody tests is not directly inferable from laboratory data obtained with sera from a small number of seroconverters. Other parameters as reactivity with virus subtypes and test precision may also substantially influence overall test sensitivity, which can be calculated only from clinical data. For the analysis of test precision and consistency, human monoclonal cocktails are used in addition to polyclonal sera. To improve the virus safety of blood products, serological tests are validated in our institute for the detection of viral markers in plasma pools and final products. Neopterin ELISAs are evaluated with prescreened patient serum pools of different neopterin concentrations. In our hands, there was no interference of haemolytic and lipaemic sera with neopterin ELISAs, but for one test, neopterin concentration were over-estimated in plasma compared to the corresponding serum samples. For two sera from patients with mamma carcinoma, higher neopterin levels (<400 nmol/l) found with one screening ELISA could not be confirmed with a reference test, where <10 nmol/l were detected. These obviously false positive results are caused by unidentified interfering substances.

Immune Activation in Patients with Alzheimer's Disease

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In 24 patients with Alzheimer's disease (8 males, 16 females; mean age 73.1 ± 6.2 years) and 14 age-matched controls (4 males, 10 females; mean age 69.7 ± 8.8 years) we measured serum concentrations

of neopterin, soluble TNF receptors (sTNFR), soluble interleukin-2 receptors (sIL-2R), immunoglobulins A, M and G as well as a panel of routine laboratory tests. In addition, we determined kynurenine and tryptophan concentrations in patients *and calculated a ratio of kynurenine/tryptophan* as an estimate for the activity of indoleamine-(2,3)-dioxygenase, which is an interferon- γ -inducible enzyme. Compared to controls, IgA ($p < 0.05$), CD23 ($p < 0.004$), sTNFR (4.6 ± 2.0 pg/ml; $p < 0.001$), sIL-2R ($p < 0.003$) and neopterin (16.2 ± 10.3 nmol/l; $p < 0.001$) were increased in patients with Alzheimer's disease. Circulating immune complexes appear to be lower in patients than in controls, the difference however was not significant. Significant correlations existed between neopterin concentrations and sTNFR ($r_s = 0.649$; $p = 0.004$), sIL-2R ($r_s = 0.588$, $p < 0.01$) and the kynurenine/tryptophan ratio ($r_s = 0.716$, $p = 0.001$). The kynurenine/tryptophan ratio also correlated with sTNFR ($r_s = 0.771$, $p = 0.001$) and sIL-2R ($r_s = 0.634$, $p = 0.005$). In addition, neopterin levels correlated with minimal state examination ($r_s = 0.65$; $p < 0.01$).

Our data show increased concentrations of immune activation markers in serum of patients with Alzheimer's disease. Increased neopterin concentrations together with increased sTNFR and sIL-2R imply a chronic state of cellular immune activation in patients and support the hypothesis of immune-mediated mechanisms as part of the pathogenesis of Alzheimer's disease. Significant degradation of tryptophan is measurable in sera of patients which is most likely due to increased endogenous formation of cytokines like interferon- γ , known to induce degradation of tryptophan. Increased degradation of tryptophan could be theoretically involved in neurological abnormalities because, on the one hand, a resulting decrease of tryptophan may diminish formation of 5-hydroxy-tryptamine (serotonin), on the other hand, products such as quinolinic acid and kynurenic acid which are formed from tryptophan upon degradation are known as potent neurotoxins. Also the increased formation of neopterin derivatives in patients with Alzheimer's disease could be of pathogenetic importance, increased production of neopterin derivatives may interfere with effects mediated by oxygen-free radicals.

Activated Immune System in Patients with Huntington's Disease

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Huntington's disease is an autosomal dominant neurodegenerative disease which is characterized by psychiatric disorders, dementia and involuntary movements. The typical onset of Huntington's disease occurs in the fourth and fifth decade of life. During recent years much has been learned about the molecular genetics of the disease, however, the exact pathogenetic mechanisms being involved remained unclear. We were interested in a potential role of the immune system in the development of Huntington's disease. Therefore, we studied concentrations of neopterin, soluble TNF receptors (sTNFR), soluble interleukin-2 receptors (sIL-2R), immunoglobulins A, M and G as well as a panel of routine laboratory tests in sera of 12 patients with Huntington's disease (8 males, 4 females, mean age 42.4 ± 11.7) and in 11 age-matched controls (7 males, 4 females, mean age 47.0 ± 12.0). In addition, we determined kynurenine and tryptophan concentrations in patients, because abnormalities of tryptophan metabolism, especially the accumulation of quinolinic acid, have been implicated to be involved in the neurodegeneration observed in Huntington's disease. A ratio of kynurenine/tryptophan was calculated to allow an estimate for the activity of indoleamine-(2,3)-dioxygenase, which is an interferon-g-inducible enzyme. Therefore, kynurenine/tryptophan ratio allows an estimate of the activity of endogenously formed interferon- γ .

In patients with Huntington's disease significantly higher serum levels were found for IgA ($p < 0.003$), C3 ($p < 0.02$), sTNF-R ($p < 0.02$) and sIL-2-R ($p < 0.04$) as compared to age-matched normals. Further, a trend to higher neopterin levels ($p = 0.06$) was observed. There existed a significant correlation between neopterin and tryptophan concentrations ($r_s = 0.65$; $p = 0.04$) and between neopterin and the kynurenine/tryptophan ratio ($r_s = 0.82$; $p = 0.04$). No correlation was seen between clinical staging (Mini Mental State Examination; Functional Capacity Scale; Abnormal Involuntary Movement Scale) and these parameters.

Our preliminary data indicate an activation of the immune system in patients with Huntington's disease. A significant correlation between neopterin concentrations and the tryptophan catabolism induced by cytokines may be of pathogenetic relevance. Accumulation of cytotoxic products like neurotoxins, formed in the tryptophan degradation pa-

thway, and the increased formation of neopterin derivatives interfering with oxygen-free radicals might be important in neurodegeneration in patients with Huntington's disease.

High Incidence of Influenza-Like Illness in a Young Military Donor Population with Raised Neopterin Values

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Since 1986 at our institute neopterin determination has been performed routinely on over 400000 blood donations as an additional marker to the mandatory required tests (HBs Ag, HCV Ab, ALT, Lues Ab, HIV 1,2 Ab). Neopterin is a reliable early marker of the activated immune system and previous studies have shown its benefit: a high incidence of IgM Ab to CMV, EBV and Parvovirus B 19 was found in the group among donors with elevated neopterin levels versus normal levels.

In February 1993 blood donations were drawn from 185 consecutive obviously healthy young soldiers after the usual routine examination. Routine testing revealed 42 donors with elevated neopterin (22.7%), 2 with elevated ALT (11.0%), 1 with a positive HCV ELISA (0.55%). This was an alarming high percentage of donations with high neopterin levels, because on average, only 2% to 3% have to be expected with a neopterin content above 10 nmol/l.

A further physical examination was performed and blood samples were drawn for extended laboratory testing. The following clinical results were obtained:

- 29 with influenza and influenza-like symptoms
- 7 with rhinitis (1 with herpes simplex labialis additionally)
- 4 had no symptoms
- 2 were absent and could not be reexamined

3 of the 42 donors with elevated neopterin had to stay in the military hospital due to serious influenza (vs. 4 of 143 with normal neopterin values). Extended laboratory testing consisted of erythrocyte sedimentation rate, β_2 -microglobulin, C-reactive protein, erythrocyte count, leucocyte differentiation count and liver enzymes (AST, ALT, gamma-GT, alkaline phosphatase). The following abnormalities were found: 14 donors with raised erythrocyte se-

dimentation rate 8 donors with raised β 2-microglobulin 5 donors with raised C-reactive protein 6 donors with raised liver enzymes 23 donors with pathological leucocyte differential count 6 donors with raised neopterin level only. We conclude that physical examination and anamnesis not always detect blood donors with fresh infections and neopterin testing reduces the risk of transfusing blood from freshly infected individuals. Furthermore, neopterin measurement by means of enzyme-linked immuno sorbent assay (ELISA), radioimmunoassay (RIA) and high performance liquid chromatography (HPLC) is well established and a powerful tool for blood transfusion services.

The Dry Eye Syndrome, Neopterin, and CMV Infection - Associated Immunological Parameters in Renal Transplantation

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Following successful renal transplantation patients often complain of symptoms characteristic for "dry eye syndrome" and show clinically bilateral reddening of their eyes. On the other hand the incidence of viral infections, esp. of CMV infections is increased in transplanted and immunosuppressed patients.

Therefore a study was undertaken first to investigate the incidence of the dry eye syndrome in renal transplant patients and second to look for etiologic factors of the keratoconjunctivitis sicca, esp. in respect to CMV infections.

22 patients (12 female, 10 male, mean age 53.4 ± 12 yrs) were investigated. Their mean duration of hemodialysis treatment was 38.8 ± 40 months before kidney transplantation. The investigation was performed on average 49.6 ± 11 months after organ grafting. In addition to the routine ophthalmological examinations in all patients a Schirmer's test (with local anaesthesia), a tear break-up time (BUT), an impression cytology of the conjunctivae and a PCR-investigation of the tear fluid was performed. As parameters of the immune response serum (S) and urine (U) neopterin (NEOP), serum amyloid A (SAA), lymphocyte subsets (LS), and serum immunoglobulins (S-IgM, S-IgG, S-IgA) were measured. In the screening for viral infections serological and

direct immunological tests were undertaken in particular for the viruses of the herpes group (S-IgM, S-IgG, S-IgA titers, U-PCR and S-PCR and in-situ hybridization).

41% (n=9) of patients complained of the characteristic dry eye symptoms (pruritus, burning and gritty sensations in the eyes), 91% (n=20) of all patients examined had pathologic changes of the outer eye. An abnormal Schirmer's test (<5 mm) was found in 57%, a shortened BUT (<10 sec) was measured in 76%, and a pathologic impression cytology ($>$ stage II) was seen under the microscope in 72% of all patients. All tear probes were negative for CMV DNA detection by PCR. The immunologic parameters U-NEOP, S-IgM, S-IgG, and S-IgM CMV titers showed a significant positive correlation ($r=0.9$, $p<0.001$). But these markers did not correlate with either of the ophthalmologic tests. Whereas the group of patients who had both, a shortened BUT and a pathologic impression cytology (n=11), showed in comparison with the patients who had less severe changes (n=11) significantly higher levels for the following markers: U-NEOP (413 ± 215 versus 174 ± 131 μ mol/mol creatinine), S-IgG (15 ± 4 versus 11 ± 2 g/l), and S-IgM CMV (107 ± 131 versus 35 ± 56). These differences between the two groups were statistically significant (Student's t-test, $p<0.01$).

In conclusion : 1. Renal allograft patients have an extremely high incidence of the dry eye syndrome. 2. These changes, in particular the consistency of the tear fluid and the morphology of the conjunctivae, are associated with increased immunological activity probably due to persistence of CMV infections.

Streptococcal Erythrogenic Toxins Induce the Formation of Neopterin in Human Peripheral Blood Mononuclear Cells via Interferon- γ

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Streptococcal erythrogenic toxins have been implicated in the pathogenesis of a toxic shock-like syndrome and of scarlet fever. These exotoxins are acting as a "superantigen" : In the presence of antigen presenting cells like macrophages, bearing

MHC class II antigen, they polyclonally stimulate as many as one of ten T cells, triggering an out of control production of cytokines like IL-2, IL-3, IL-6, tumor necrosis factor- α and interferon- γ . In contrast to this, common antigens stimulate only one in 10000 of the T cells on a clonal level. In this study, we tested whether exposure of human monocytic cells to streptococcal erythrogenic toxins A, B, C, and a streptococcal-derived Mitogen BX is associated with synthesis of neopterin in vitro. We found that streptococcal erythrogenic toxins A, B, C, and Mitogen BX induced interferon- γ and neopterin production in peripheral blood mononuclear cells of three healthy individuals. Neopterin production was not induced, when the human myelocytoma cell line THP-1 was stimulated with these toxins, and there was only a slight costimulatory effect of streptococcal erythrogenic toxin A together with interferon- γ stimulation.

From our investigations we conclude that there is no direct effect of streptococcal erythrogenic toxins on neopterin production. However, in peripheral blood mononuclear cell culture, these toxins were as potent inducers of neopterin production as interferon- γ indicating that neopterin production in toxin-treated peripheral blood mononuclear cells is mediated via interferon- γ . Neopterin concentrations might turn out to be an indicator for disease development in bacterial infections involving superantigens.

Effect of Neopterin-Derivatives on Hydrogen Peroxide-Induced Luminol-Dependent Chemiluminescence: Mechanistic Aspects

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Neopterin, 6-D-erythro-1',2',3'-trihydroxypropylpterin, and its dihydroform, 7,8-dihydroneopterin, are synthesized by human monocytes/macrophages upon stimulation by interferon- γ . In the presence of iron chelator complexes neopterin enhances hydrogen peroxide-induced luminol-dependent chemiluminescence at neutral or slightly alkaline pH = 7.5. In contrast, 7,8-dihydroneopterin scavenges chemiluminescence independently from the pH value and iron. In this study, we explored in more detail the mechanism possibly involved: analysis of

the reaction products shows that 7,8-dihydroneopterin is oxidized and degraded to 7,8-dihydroxanthopterin and xanthopterin, whereas the neopterin molecule is not chemically altered during the chemiluminescence reaction. Investigations of the neopterin-induced effect show that mannitol, a scavenger of hydroxyl radicals, does not alter the enhancing effect of neopterin. L-histidine, which scavenges singlet oxygen almost as effective as hydroxyl radicals, reduces the enhancing effect of neopterin. However, singlet oxygen was not detectable during the reaction by measuring monomol light emission (1270 nm). When replacing hydrogen peroxide by 3-morpholinocydonimine, a generator of hydroxyl radicals, or naphthalene-endoperoxide, a generator of singlet oxygen, in the luminol-dependent chemiluminescence assay, neopterin shows no enhancing effect irrespective of the presence of iron-(III)-EDTA.

The data suggest that neopterin enhances hydrogen peroxide-induced luminol-dependent chemiluminescence in the presence of iron-(III)-EDTA in a catalytic manner. It catalyzes the formation of reactive oxygen species from hydrogen peroxide, but other species than singlet oxygen or hydroxyl radicals appear to be important in this reaction.

7,8-Dihydroneopterin Inhibits Low Density Lipoprotein Oxidation in Vitro

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Pterin, neopterin and 7,8-dihydroneopterin were tested for their ability to interfere with the Cu^{2+} - and 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation of low density lipoprotein. While pterin and neopterin did not exert any effect, 7,8-dihydroneopterin dramatically increases, in a strictly dose-dependent manner, the lag time of the oxidation reaction. This observation was also made when linoleate was studied as a model for unsaturated fatty acids. The kinetics of the oxidation inhibition suggests that 7,8-dihydroneopterin is a potent chain breaking antioxidant which functions by scavenging lipid peroxy radicals. The activity of 7,8-dihydroneopterin in this respect is at least as large as that of the well-known antioxidant tocopherol; the half life of tocopherol in a low

density lipoprotein oxidation assay is strongly prolonged by adding 7,8-dihydroneopterin to the system. From analysis of the fluorescence behaviour of the reaction products, chemical reaction of 7,8-dihydroneopterin to either neopterin or, more probably, xanthopterin seems to take place during its interaction with the lipid peroxy radicals. The biological consequences of our findings still have to be established.

Parallel Changes of Biopterin and Melatonin in Serial Transplants of DMBA- Induced Mammary Tumors in Fischer Rats. Interactions between the Pineal Gland and Cellular Immunity in Malignancy ?

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Nocturnal (23 : 00–07 : 00 h) urinary total biopterin (after acidic oxidation of reduced biopterins) and melatonin were analyzed during the growth of two passages of a mammary tumor cell line in female F344-Fischer rats. In addition, nocturnal (02 : 00–03 : 00 h) peak concentrations of pineal melatonin in plasma were analyzed when tumors had reached comparable average volumes of 25–30 ml.

As tetrahydrobiopterin is produced by murine macrophages (and other cells) mainly in response to interferon- γ released by activated T lymphocytes, measurement of biopterin can be used to estimate the state of cellular immunity. At passage 2 (the tumor presented as slow growing localized carcinosarcoma), biopterin showed a progressive increase during tumor growth and reached more than 200% ($p < 0.05 - 0.005$) of controls. Urinary and plasma melatonin were elevated by 30–50% ($p < 0.05$) and 42%, respectively. At passage 12 (the tumor presented as fast growing metastasizing sarcoma), both urinary biopterin and melatonin were lower than controls by about 20–30% ($p < 0.05$ and < 0.025 , respectively); plasma melatonin was depleted by 70% ($p < 0.005$). The parallel changes of both parameters observed at each tumor passage point to a close link between the pineal hormone melatonin and cellular immunity. The opposite trends observed at the two passages indicate a clear stimulation of the immune system and the pineal gland at early but inhibition at advanced stages of cancer.

Xanthopterin and Lipid Metabolism

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Our previous observations have shown that both, kynurenine and neopterin, induce changes of fatty acid incorporation into phospholipids as well as increase of cholesterol and decrease of phospholipids content in samples used for phospholipid biosynthesis in vitro. Kynurenine and neopterin have been found in mammalian tissue whereas xanthopterin is more characteristic for insects, amphibia and fish.

In this study, we tested whether xanthopterin has a similar effect. Incorporation of fatty acids into phospholipids has been investigated in homogenized liver tissue of 10 male white Wistar rats according to previously described methods. Xanthopterin (4 and 20 nmol/g wet weight) was added into experimental test tubes before the first incubation (4 hours at 37°C) in order to investigate a possible effect of this substance. After the first incubation, 60 μ mol of 20% fatty acid solution in chloroform and 40 μ mol of glycerine were added to each test tube (control and experimental). All test tubes were incubated at 37°C for another 4 hours. Thereafter, 7.5 ml of chloroform-methanol mixture 2 : 1 was added to each sample. The samples were mixed and left for 40 min. at room temperature. Then the chloroform-methanol extract was filtered and the filtrate was carefully treated according to the method of Folch and others. The whole cholesterol concentration, the amount of phospholipids as well as fatty acid content of phospholipids was determined by the methods previously described.

We observed that xanthopterin did not change the fatty acid incorporation into phospholipids as well as the content of cholesterol and phospholipids in samples. Further studies are required for the investigation of other pteridines to possibly interfere with lipid metabolism.

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Association between the Renin-Angiotensin System and Immune Activation in Vitro and in Vivo

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Both, the renin-angiotensin and the immune system, are involved in the pathogenesis of certain human diseases, e.g. sarcoidosis, vasculitis and congestive heart failure (CHF). We recently demonstrated an influence of the angiotensin-converting enzyme (ACE) inhibitor Captopril (Bristol-Myers Squibb) on interferon- γ (IFN- γ)-mediated macrophage activation in vitro. Similarly, IFN- γ can effect ACE activity in macrophages. This interaction is not well understood and may occur through a prostaglandin-dependent mechanism. The aims of this study were a) to investigate an eventual effect of indomethacin on macrophage activation after stimulation with a combination of IFN- γ and Captopril, and b) to analyze a possible association between ACE, plasma renin activity and markers of immune activation in CHF.

For the experiments, the human myelomonocytic cell line THP-1 that behaves similarly to freshly isolated peripheral blood monocytes/macrophages was used. Cells were seeded at 6×10^5 cells/ml in 24-well plates and treated with various stimuli for 72 hours. Captopril (2.5, 1.25 mg/ml) was applied either alone or in combination with Indomethacin (3 mmol/L) in absence or presence of 250 units/ml IFN- γ . Radioimmunoassays were used for measurement of neopterin (Henning-Berlin, Germany), ACE (Buhlmann, Switzerland) and plasma renin activity (CIS-biointernational, France). Serum levels of soluble tumor necrosis factor (sTNF-R) and interleukin-2 (sIL-2R) receptors were determined by ELISAs (T Cell Diagnostics, USA). Indomethacin did not influence the increased macrophage activation and neopterin production which was induced by the combination of Captopril and IFN- γ . Therefore, the influence of Captopril on IFN- γ -mediated effects appears not only to be linked with prostaglandin synthesis but also with special ACE binding sites on macrophages.

To further confirm the results of the in vitro studies, 52 patients with CHF (mean age 44.2 ± 12.1

years; mean NYHA class 2.16 ± 0.9) were investigated for associations between the immune system and the renin-angiotensin system. The mean concentrations of neopterin, sTNF-R and renin were significantly higher in patients with NYHA classes 3-4 compared to patients with NYHA classes 1-2 ($p < 0.05$). No such difference existed for sIL-2R. Neopterin levels correlated with ACE ($r_s = 0.33$, $p < 0.05$; Spearman rank correlation analysis), and sTNF-R levels with plasma renin activity ($r_s = 0.39$, $p < 0.05$).

The study demonstrates an association between the renin-angiotensin system and activation of macrophages in vitro which can be confirmed to some degree also in patients with CHF. The data suggest that specific cytokines are involved in the development and progression of CHF.

Correlation Between Hepatitis A-IgM Antibodies and Raised Neopterin Concentrations in Patients and Blood Donors

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In former studies and in the last workshop we could present data of an increased prevalence of IgM antibodies to cytomegalovirus, Epstein-Barr Virus and parvovirus B19 in blood donations with above-normal neopterin concentration. Neopterin is known to be a sensitive marker in hepatitis A, too. Former studies demonstrated a correlation between acute hepatitis A with IgM antibody-positivity and raised neopterin concentrations. These results could be confirmed in our laboratory: In the last year we tested 3 adults (between 19 and 33 years old) and 9 children (3-17 years) who suffered from acute hepatitis A. All of them were hepatitis A IgM-positive, all of the adults and 7 of the 9 children had high neopterin levels, only 2 of the children lay below but very close to the cutoff. Mean neopterin concentration of the adults: 30.3 nmol/l, of the children: 19.6 nmol/l. Considering these results and reports about transmission of hepatitis A by factor VIII concentrates treated by a virucidal method we took a look on blood donations in our institute: 783 blood donations with elevated neopterin concentrations (mean 12.8) and 388 donations with neopterin concentrations below the cutoff of 10 nmol/l (mean 6.0) were tested for hepatitis A IgM antibodies: Neither in the neopterin-elevated donor

group nor in the control group we could find hepatitis A IgM antibodies. Western Austria shows a decreasing prevalence of hepatitis A antibodies. This trend is most pronounced in the youngest adult group between 18 and 30 years, i.e. that in the most important blood donor group very seldom acute hepatitis A infections can be observed. We conclude that the chance to find acute hepatitis A infections among Tyrolean blood donors is very low. On the other hand, confirmed by our observations on patients with hepatitis A, neopterin testing on blood donations may reduce the risk to transmit hepatitis A by blood transfusion to zero.

Neopterin-Induced Suppression of Erythropoietin Production in Vitro

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Elevated concentrations of neopterin are described in a variety of diseases with activation of cellular immune response. As previously shown serum concentrations of neopterin were inversely correlated with hemoglobin and the degree of anemia in patients with hematological and malignant disorders. It was speculated, that in these cases pteridines could be involved in the reduced red cell production. As one possible mechanism for impaired erythropoiesis we hypothesize that neopterin is able to suppress the production of erythropoietin (EPO). To test this hypothesis the influence of neopterin on EPO-formation in con-fluent cultures of the human hepatoma cell line HepG2 was investigated. Results are shown in the table. Data are means $\pm$ SD of EPO-production from eight separate cultures during a 24th experiment. P values indicate differences compared with control cultures (Dunnett's test).

Neopterin (mmol/l)	EPO (U/g cell protein)	p
0 (control culture)	318.5 $\pm$ 55.2	
3	324.6 $\pm$ 42.4	n.s
10	305.0 $\pm$ 47.2	n.s
30	291.7 $\pm$ 36.3	n.s
100	207.5 $\pm$ 27.9	<0.01
300	135.1 $\pm$ 35.1	<0.001

The result of this study confirm our hypothesis

of a neopterin-induced suppression of EPO-formation in HepG2 cultures in a dose dependent manner. This is the first report of direct involvement of neopterin on cellular regulation of EPO-production. Thus we speculate that under in vivo conditions associated with activation of macrophages anemia can worsen by high concentrations of neopterin.

The Design of High Performance Assays for Small Molecules

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Nowhere better than in the immunoassay of metabolites are the limitations of immunoassay seen. The specific determination of very low concentrations of small, closely-related compounds can often not be met by current competitive-format immunoassay systems.

While significant advances have been made in the immunoassay of large molecular weight analytes, enabling great benefit to be drawn from the application of intense labels such as provided by Enzyme Amplification, improvements in the performance of small molecular weight analytes have been much more modest. Largely, this is because immunoassays for large molecules may be formatted such that it is the fraction of capture antibody binding analyte which is determined, whereas in contrast, with small analytes, where competitive format immunoassay are employed it is the fraction of antibody which has not bound analyte which is measured. Not surprisingly, this causes high imprecision at low analyte concentration.

Two general non-competitive methods have been developed to overcome this limitation. This first is the Anti-Complex Immunoassay System. In this a secondary anticomplex monoclonal antibody is produced which binds with the complex of analyte and a primary antibody against it, while not binding either component alone. This may then be labelled and added simultaneously with analyte to the primary antibody for the determination of analyte. The second method is termed the "Selective Antibody Immunoassay System". In this, analyte is allowed to bind with primary antibody and a specific blocker (such as a chemical conjugate of analyte with a protein or an anti-idiotypic monoclonal antibody) is added to block the remaining unbound primary antibody sites. Selective antibody is an antibody

which can bind those primary antibody sites bound but not those bound by blocker. The addition of labelled selective antibody then allows the determination of the analyte bound sites and thus analyte concentration.

Especially in fields such as pteridine analysis where there are closely related substances, the specificity of the immunoassay is of great importance. Again for fundamental reasons, such as being dependent on only one binding event, competitive immunoassay is also flawed in this aspect. The Multiple Binding Assay (MBA) has been designed to overcome this problem. In this, analyte is passed from one antibody to another during the analytical phase, the final specificity being the product of the specificities of the different antibodies used.

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Infection of Macaques with SIV and HIV-2: The Relevance of Neopterin for Pathogenicity and Vaccines Studies

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Neopterin is known to be a predictive marker for disease progression in man infected with the human immunodeficiency virus (HIV). When measured in urine it is a simple and noninvasive tool. We have investigated the value of urinary neopterin in the SIV (simian immunodeficiency virus)/HIV-2 macaque model not only in the early phase of infection, but also longitudinally in pathogenicity as well as in vaccine studies.

Macaques infected with different pathogenic SIVmac strains or a SIV/HIV (SHIV) hybridvirus composed of the reverse transcriptase from HIV-1 hybridvirus containing the backbone from SIV, but env and some regulatory genes from HIV-1) neo-

pterin was only 2-4-fold elevated in this early phase. When the early changes of neopterin were examined in studies with experimental SIV split or subunit vaccines, lack of or very low neopterin increase correlated with sterile or strong immunity, whereas in vaccine failures increases like in the unvaccinated controls were measured. Follow-up studies of SIV infected macaques with AIDS-related death as an endpoint revealed constantly rising high neopterin concentrations in the majority of the animals. In such animals high neopterin concentrations correlated with increased p27 antigenaemia and cell-associated viral load, but not with decrease of CD4/CD8 ratios at the time of death.

In conclusion, the degree of initial neopterin increase was predictive for pathogenicity of the different viruses used for infection of macaques. In the SIV macaque model neopterin concentrations have proven to be an early and reliable marker indicating vaccine protection. In our hands neopterin is the best predictive marker for disease progression.

Neopterin: A Reliable Basis for the Decision to Use Antibiotics in an ICU in a Trauma Center

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Infection is a major risk in intensive care Unit (ICU) patients. The early use of broad spectrum antibiotics may lead to the selection of resistant microorganisms and is thus not warranted. The hypothesis that neopterin blood levels provide a reliable basis for the decision to use antibiotics was tested prospectively in 536 traumatised ICU patients. When infection was suspected, antibiotics were given either if deemed necessary (control group, 1992) or if neopterin levels were >40 nmol/l (study group, 1993). The number of patients with infections (24% versus 29%), the number of infectious episodes per patient (3.1 versus 4.8), the incidence of infections with *Staphylococcus* and *Pseudomonas*, and the treatment costs (minus 20%) were lower in the study group. Length of ICU stay and outcome remained unchanged. We conclude that it is beneficial to base the decision to use antibiotics in ICU patients with suspected infections on neopterin testing.

Neopterin and 7,8 Dihydroneopterin together with Cyclic Guanosine Monophosphate Induce c-fos Gene

Expression

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Reactive oxygen species have been frequently implicated in the initiation and promotion of carcinogenesis. Direct effects on growth factors and other signalling pathways of both antioxidants and oxidants have recently been shown. Besides various oxidants, phenolic antioxidants were shown to specifically induce expression of c-fos and c-jun mRNAs. In humans, activation of the cellular immune system is associated with increased neopterin concentrations because large amounts of neopterin are released from human monocytes/macrophages upon stimulation with interferon- γ . In parallel, production of 7,8-dihydroneopterin occurs. Recent data suggest that the formation of neopterin and 7,8-dihydroneopterin is linked with the cytotoxic repertoire of activated macrophages because neopterin was found to be an enhancer for mechanisms mediated by reactive oxygen and chloride species whereas 7,8-dihydroneopterin is a scavenger. Interferon- γ also induces the formation of nitric oxide in a variety of mammalian cells. The thereby enhanced 5,6,7,8-tetrahydrobiopterin up-regulates the activity of inducible nitric oxide synthase. Nitric oxide is a free radical which mediates some of its effects via the formation of cyclic guanosine monophosphate (cGMP) because NO activates soluble guanylyl cyclase, most probably by binding to its heme group.

In this study, we explored the potential of neopterin 7,8-dihydroneopterin and cGMP on c-fos gene expression. NIH3T3 mouse fibroblasts were stably transfected with a c-fosCAT construct containing the 5'-flanking sequence of the c-fos gene up to position -711. Expression of the c-fos-CAT construct was stimulated by the known inducer of c-fos transcription phorbol 12,13-dibutyrate (PDBu). The simultaneous application of 7,8-dihydroneopterin (400 nM) and cGMP (20 μ M) led to a significant transcriptional activation of the c-fosCAT reporter construct. A similar but less pronounced effect could be achieved by using neopterin and 7,8-dihydroneopterin. The direct effect of neopterin and 7,8-dihydroneopterin on c-fos transactivation was tested in the same experiment. However, the direct transactivation of the c-fosCAT reporter construct was marginal in comparison to the influence

of the combination of neopterin derivatives with cGMP. In humans, interferon- γ induces the release of neopterin and 7,8-dihydroneopterin and also the synthesis of the nitric oxide radical in other cell types which in turn stimulates formation of cGMP. Thus, in certain situations all three substances, namely neopterin, 7,8-dihydroneopterin and cGMP, may be present locally and even in the circulation at the same time. This constellation would significantly enhance the risk of c-fos gene expression and therefore promote tumor growth and development. The prognostic value of higher neopterin concentrations to predict disease progression and death in malignant diseases could be related to the capacity of neopterin derivatives to induce oncogene expression. The endogenous formation of cytokines at local sites of inflammation could be of importance within this context.

Effect of NADH on Biosynthesis of Dopamine and Tetrahydrobiopterin in Rat PC-12 Cells

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As we know that the classical treatment of patients with Parkinson's disease with L-DOPA leads to a depression of the endogenous dopamine biosynthesis, we considered a new concept to overcome the dopamine deficit in the basal ganglia of patients. Our new strategy was to stimulate the endogenous L-DOPA biosynthesis via activation of the key enzyme tyrosine hydroxylase. The activity of this enzyme as well as the concentration of its cofactor tetrahydrobiopterin are reduced in the basal ganglia and therefore, NADH which is cofactor of dihydropyridine reductase, should lead to an increase in tetrahydrobiopterin synthesis. This increase in turn should stimulate the endogenous L-DOPA biosynthesis by tyrosine hydroxylase.

As a model system for investigation of the reliability of this concept, rat PC12 cells, derived from a malignant tumor of the adrenal medulla, were used. We found a concentration- and time-dependent increase of dopamine biosynthesis in these cells after incubation with an NADH-enriched medium as well as a 75% increase of tyrosine hydroxylase activity. These experiments showed that

NADH acts on tyrosine hydroxylase directly.

After treatment of Parkinsonian patients with NADH for 14 days, they showed an improvement of disability of about 20%. In parallel, an increase of urinary homovanillic acid was observed, indicating higher dopamine turnover. In preliminary experiments, we measured the biopterin concentrations in PC12 cell homogenates after incubation with different NADH concentrations. After acid and alkaline oxidative pretreatment of the homogenates, we found that essentially all biopterin is present in the tetrahydro form. The employed NADH solutions were free of tetrahydrobiopterin. In these experiments, we detected a moderate stimulation of tetrahydrobiopterin synthesis. Due to the preliminary nature of these experiments, further investigations are required for a definitive conclusion. In particular, experiments are planned in order to establish a potential effect of NADH on GTP cyclohydrolase I.

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Effects of Neopterin and 7,8-Dihydroneopterin on Luminol-Dependent Chemiluminescence Induced by Reactive Intermediates Formed from Hydrogen Peroxide, Chloramine-T, Hypochloride and Nitrite

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Phagocytes like granulocytes and monocytes/macrophages when stimulated, release toxic reactive oxygen metabolites (ROMs) such as hydrogen peroxide, nitrite, peroxonitrite, superoxide anion and hydroxyl radical to the cellular environment as well as other oxidants such as N-chloramine and hypochlorous acid derived from myeloperoxidase. Upon stimulation by interferon- γ , human macrophages synthesize and release large amounts of 7, 8-dihydroneopterin and neopterin. Previous studies have shown that neopterin enhances the chloramine-T-induced chemiluminescence and the hydrogen peroxide-induced chemiluminescence in the presence of iron-(III)-chelator complex. In contrast, dihydroneopterin scavenges the effect in both cases, independent from the presence of iron-(III)-EDTA.

Endogenously formed neopterin derivatives may thus serve as regulators of the cytotoxicity of ROMs in vivo. In the present work, we investigated the effect of neopterin (50 μ M) and dihydroneopterin (50 μ M) on oxidant systems using hypochloride (NaOCl : 3 pM) in comparison to chloramine-T and hydrogen peroxide +/– nitrite (H₂O₂ : 2 M; NaNO₂ : 2 mM) in the presence or absence of Fe-(III)-EDTA (5 μ M) at pH=7.5.

Both, the enhancement by neopterin and the scavenger effect of dihydroneopterin, are found for the hypochloride-induced chemiluminescence, but the influence of neopterin on reactive species derived from hypochloride is much higher than that in the chloramine-T-system (0.3 mM). Examination of the kinetics show a gradually rising and longer-lasting enhancer effect in the chloramine-T-system, whereas in the hypochloride-system the reactive species are formed quickly but only for a short period. Secondary metabolites in the chloramine-T-system appeared to lengthen the half-life of the ROMs sensitive to pteridines.

Chemiluminescence induced by the ROMs from hydrogen peroxide + nitrite similar as the ROMs from hydrogen peroxide are scavenged by dihydroneopterin and neopterin, whereas in the presence of iron-(III)-EDTA, neopterin acts as an enhancer. The chemiluminescence induced from hydrogen peroxide-formed ROMs in the presence of nitrite is more enhanced than the chemiluminescence induced from the hydrogen peroxide system.

When investigating the influence of histidine (10 mM), mannitol (10 mM), catalase (25 U/ml) and superoxide dismutase (25 U/ml), only catalase has an additional positive effect on neopterin with regard to its enhancer role in the hydrogen peroxide-system and the hydrogen peroxide nitrite-system. In the presence of iron-(III)-EDTA, catalase does not rise the signal activity of the luminol reaction. These data imply that neopterin impedes the effect of catalase by interfering with the iron-(III)-EDTA complex.

Catalase also increases the scavenger effect of dihydroneopterin in the hydrogen peroxide system and the scavenger effect of dihydroneopterin in the hydrogen peroxide-nitrite-system is also activated by histidine.

In conclusion, neopterin and dihydroneopterin react with ROMs which are produced and released in a large amount by a variety of human cells. Pterins endogenously formed during immune response mechanism may contribute to up- or down-regulate the cytotoxicity of the above-mentioned

toxic agents. Interaction between various reactive intermediates such as hydroxyl radicals, singlet oxygen and nitric oxide appear to be important.

Nitrate Formation as Predictive Parameter for Acute Graft-versus-Host Disease after Human Allogeneic Bone Marrow Transplantation

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Since evidence has accumulated concerning a putative role of nitric oxide (NO) in graft-versus-host disease (GvH-D) we performed follow up measurements of the stable endproduct of NO, nitrate (NO_3^-), in serum of patients undergoing allogeneic ($n=16$) and autologous ($n=6$, as a control) bone marrow transplantation and compared them with serum levels of soluble tumor necrosis factor 75 (sTNFrec 75), neopterin, both them known as sensitive indicators of cellular immune activation phenomena involving macrophages, and interleukin-10 (IL-10), a major TH-2 cell-derived cytokine and potent inhibitor of macrophage activation and NO formation. A significant rise in nitrate levels was observed in patients with GvH-D and preceded the onset of clinical symptoms by up to three days. In contrast to indicators of macrophage activation, i.e. neopterin and sTNFrec 75, nitrate concentrations were not significantly altered from baseline levels during infectious complications, as nitrate concentrations did not fluctuate in patients after autologous engraftment. During episodes of acute GvH-D, nitrate concentrations were strongly positively correlated to serum neopterin and sTNFrec 75 levels but also significantly related to IL-10, while otherwise a negative correlation between IL-10 and nitrate concentrations was evident. Therefore, nitrate determination could be a useful early indicator of the initiation of human GvH-D. Our results provide some further insights concerning cytokine function and macrophage activation in the course of human GvH-D in vivo which could give some useful hints for the development of new therapeutic approaches for this disease.

Formation of Neopterin from 7,8-Dihydroneopterin Derivatives in Vitro and in Vivo

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Cytokines like interferon- γ and tumour necrosis factor- α stimulate tetrahydrobiopterin synthesis by induction of GTP cyclohydrolase I in many cells. In human cells, particularly in human macrophages, 7,8-dihydroneopterin triphosphate accumulates upon induction of GTP-cyclohydrolase I due to a low 6-pyruvoyl tetrahydropterin synthase activity. In cells as well as in body fluids, neopterin derivatives occur in addition to 7,8-dihydroneopterin, although dihydroneopterin is not oxidized to neopterin at physiologic pH.

In this work, we investigated the relationship of 7,8-dihydro- and fully aromatic neopterin species. We compared concentrations of dihydro- and fully aromatic species after incubation at physiologic pH of synthetic 7,8-dihydroneopterin, 7,8-dihydroneopterin monophosphate as well as 7,8-dihydroneopterin triphosphate generated by recombinant *E. coli* GTP cyclohydrolase I. In protein fractions of stimulated THP-1 cells, we incubated 7,8-dihydroneopterin with NAD and NADP, neopterin with NADH and NADPH to check for enzymes altering the redox state of neopterin. In vivo, we monitored the ratio of 7,8-dihydroneopterin and neopterin in the course of interferon-treatment of patients, as well as in a sample of 98 patients with rheumatoid arthritis. We found that in sera the ratio of neopterin to 7,8-dihydroneopterin does not change during interferon therapy and is found equal in patients with rheumatoid arthritis as compared to healthy controls. Whereas 7,8-dihydroneopterin is slowly converted to 7,8-dihydroxanthopterin, a considerable portion of 7,8-dihydroneopterin monophosphate and 7,8-dihydroneopterin triphosphate is readily oxidized to the respective fully aromatic neopterin phosphates. Thus, 7,8-dihydroneopterin triphosphate is oxidized aerobically to neopterin.

Upon cleavage of the phosphates by phosphatases, 7,8-dihydroneopterin no longer yields neopterin, but slowly degrades to 7,8-dihydroxanthopterin. No indications for an enzymatic interconversion of the two species and no alteration of the oxidation process by the interferon-treatment was found.

Tetrahydrobiopterin Biosynthesis in Relation to Nitric Oxide Synthase - a Short Review

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In a variety of mammalian cells, GTP cyclohydrolase I activity is stimulated by treatment with cytokines such as interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α). This leads to increased formation of tetrahydrobiopterin and, depending on cell type and species, of neopterin. In macrophages and a number of other cell types, nitric oxide synthase (NOS) is induced by the same cytokines in parallel. As is now established, NOS occurs in various isoforms. High-output NOS, which is induced by cytokines, is easily detectable in cells of rodent origin but can also be found in human cells using specialized regimens of treatment. For macrophages, NO produced by inducible NOS is part of the antimicrobial armature. Constitutive NOS, which is activated via a pulse-like increase of free intracellular Ca^{2+} ions rather than via increased transcription and translation of the NOS gene, is found in neuronal cells and as a myristoylated isoform in endothelial cells.

In these tissues, NO plays a role as neurotransmitter and as a vasodilator. Using murine dermal fibroblasts, human umbilical vein endothelial cells and human cervix carcinoma cells ME-180 which express a neuronal NOS, we demonstrated that the activity of all three isoforms is stimulated by increasing intracellular tetrahydrobiopterin levels and is inhibited by depleting cells of the cofactor. Thus, the availability of tetrahydrobiopterin is one of several possible ways to regulate activity of NOS. The exact role of tetrahydrobiopterin in the NOS reaction, however, is not yet fully understood. The current view on this issue is that tetrahydrobiopterin acts as a hydroxylating cofactor and as a stabilizer of NOS enzyme structure. As we showed recently, NOS expression is strongly increased by reducing free iron levels within the cell but is inhibited by increasing iron concentrations. This is not mediated via interference with tetrahydrobiopterin but is caused by changing NOS mRNA transcription. Studying expression of NOS at transcriptional and translational level, an interference of DAHP and/or sepiapterin was found which varied depending on the cell type tested. These results suggest that under certain circumstances drugs altering tetrahydrobiopterin biosynthesis may act not only on NOS activity but also on NOS expression itself.

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Protection of Pteridines

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The low solubility of pterins and lumazines limits chemical syntheses and structural modifications to large extent. Protecting groups overcome these difficulties due to reduced intermolecular aggregation of amino- and hydroxy function. We have shown that the introduction of the p-nitrophenylethyl (NPE) group via a Mitsunobu reaction is a versatile approach to block amide functions. N²-Acetylpterins are converted into 2-acetyl-amino-4-p-nitrophenylethoxypteridines but treatment of 2-dimethylaminomethylpterins in an analogous manner led selectively to N-3 substitution.

This synthetic approach was extended to various derivatives to study the influence of substituents during Mitsunobu alkylations. 2-Dimethylamino-(1), 2-methylamino-(2) and 2-methylthio-4(3H)pteridone (3) give O-alkylation in 84 (4), 72 (5) and 72% (6) yield, respectively. Methylations, however, of 2 with MeOH under Mitsunobu conditions led to a 1 : 1 mixture of the O⁴ (7) and N-3-methyl (8) derivatives.

In an attempt to convert 5,6,7,8-tetrahydrobiopterin (9) into 2,4-diamino-6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine (10) N², N⁵, 1'

2-tetraacetyltetrahydrobiopterin (11) was subject to the Mitsunobu reaction with p-nitrophenylethanol, MeOH and EtOH, respectively, in order to achieve O⁴-alkylation analogous to the model reactions.

However, 11 was alkylated neither at O⁴ nor at the exocyclic amino group to form 12-14. It is most likely that the N⁵-acetyl sterically hinders O-alkylation which was expected from the directing power of the N²-acetyl group.

Acylation of 5-acetyl-5,6,7,8-tetrahydrobiopterin by isobutyric and pivalic anhydride, respectively, led to more bulky substituents at the 2-amino group which could, however, not prevent alkylation in an analogous manner at the acylamino function. Diacylation of the 2-amino group by o-phthalic anhydride may solve in future studies this problem.

Yao Q, Pfeleiderer W : Pteridines, 1994; 5 : 90

Correlation between Cell Free Viral Load, Neopterin and Disease Outcome in Vertically HIV-1 Infected Children

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To date the major prognostic parameter for progression in HIV-disease, is the concentration of CD4 receptor expressing T cells in peripheral blood. We hypothesized that increased cell-free viral load or secreted activation markers could predict subsequent decline in CD4 and guide therapeutic decisions.

We measured the concentration of HIV ICD-p24 antigen (Dupont Elisa), RNA-PCR (Roche Reverse transcriptase), and neopterin (Henning, RIA) in serially collected serum/plasma samples from 49 vertically HIV-1 infected children, aged 6-86 months. We compared the measures of extracellular viral load to levels of neopterin, CD4 concentration and clinical measures.

The concentration of cell-free HIV as measured

by ICD-p24 antigen or RNA-PCR was highly predictive for a fall in CD4 -cell concentration during the subsequent 6 months. Also, whereas ICD-p24 antigen and RNA-PCR directly correlated ($r=0.4$, $p<0.01$; repeated measures regression), neopterin levels correlated only with RNA-PCR. Measures of viral load as well as neopterin levels were predictive of development of encephalopathy (ICD-p24 antigen : $p=0.01$; OR 1.43; RNA-PCR : $p=0.0006$; OR =1.76; neopterin : $p=0.02$; OR= 1.37). In addition, increased neopterin correlated with the development of lymphoid interstitial pneumonitis (LIP; $p=0.026$, OR=1.27). Zidovudine decreased viral burden as measured by ICD-p24 antigen and RNA-PCR ($63 \pm 21.3\%$ and $69 \pm 18.7\%$, mean $\pm$ SD, respectively).

The likelihood of subsequent decline in CD4 concentration increased with higher cell-free virus. Neopterin reflects an acutely activated immune system and was found to be elevated in LIP as well as under circumstances such as encephalopathy in which cell-free viral load was high.

We conclude that serial measures of viral load may be helpful in judging prognosis and therapeutic efficacy.

Immune Activation in the Pathogenesis of HIV Infection

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Abnormalities affecting both the cellular and the humoral part of the immune system have been described in human immunodeficiency virus (HIV) infection. The immunodeficiency is mainly due to numerical and functional defects of the CD4+ T-cell subset. However, immune activation characterizes a substantial part in HIV infection, and signs of immune activation such as increased concentrations of cytokines in serum and/or other body fluids occur early in the course of the infection. Interferon- γ , interleukin-6 and tumor necrosis factor (TNF)- α have been reported to be increased in many individuals with HIV infection. In addition, HIV infection is characterized by increased serum levels of many products of immune activation, e.g., neopterin and β 2-microglobulin.

In a longitudinal study, we assessed whether the

decline in the CD4+ T-cell count is associated with increased concentrations of soluble intercellular adhesion molecule 1 (sICAM-1) and soluble tumor necrosis factor receptor 75 (sTNFR 75), compared to increased concentrations of β 2-microglobulin and urinary neopterin. Forty-seven individuals representing all stages of HIV infection were followed-up for a mean of 12.7 months (range, 8 to 16 months). The percentage of the change of the CD4+ T-cell count from study entry to study end ranged from -97% to +98%, the median was -33%. Concentrations of urinary neopterin, sTNFR 75 and β 2-microglobulin correlated with the percentage of the change of the CD4+ T-cell count from study entry to study end ($r_s = -0.52$, $p = 0.0005$, $r_s = -0.51$, $p = 0.0006$ and $r_s = -0.46$, $p = 0.002$ respectively), but those of sICAM-1 did not. This difference was found despite significant correlations between sICAM-1 and urinary neopterin, sTNFR 75, and β 2-microglobulin. Levels of sICAM-1 obtained at study entry correlated strongly with levels of sICAM-1 obtained at study end ($r_s = 0.54$, $p = 0.001$). In a multivariate linear regression analysis urinary neopterin and sTNFR 75 were jointly significant for the percentage of the change of the CD4+ T-cell count. These results suggest that sTNFR 75 is a useful marker to estimate disease progression in HIV infection, whereas sICAM-1 does not seem to provide any information related to the decline of the CD4 T-cell count.

The causes behind the differences to predict the CD4 T-cell depletion among the immune activation markers neopterin and β 2-microglobulin and the soluble receptors sTNFR 75 and sICAM-1 may be that these molecules are produced by different cell types. Neopterin is released mainly from macrophages under the control of interferon- γ ; however, TNF- α synergistically amplifies macrophages for neopterin secretion. Previously, we found strong correlations between interferon- γ , urinary neopterin and sTNFR 75, which supports the concept that TNF- α and interferon- γ may synergistically enhance the activation on target cells. TNFR 75 is present on many cell types, especially on stimulated T and B cells; β 2-microglobulin, the constant component of the class I major histocompatibility complex, is found on all nucleated cells. Serum β 2-microglobulin levels are believed to reflect general immune activation and lymphoid cell turnover. The strong correlation found between β 2-microglobulin and sICAM-1 suggest that a more general immune activation could be the cause of increased sICAM-1 levels. Levels of the immune activation markers neopterin, sTNFR 75 and β 2-microglobulin help the clinician to define those who will lose their CD4+ T cells more rapidly which is an indicator of bad prognosis. There is a need for such information in addition to the CD4+ T-cell count to estimate prognosis in HIV infection.