Serum neopterin as an immunological marker of disease activity in inflammatory diseases

F.E.V. Hamerlinck

SERUM NEOPTERIN AS AN IMMUNOLOGICAL MARKER OF DISEASE ACTIVITY IN INFLAMMATORY DISEASES

STELLINGEN BEHORENDE BIJ HET PROEFSCHRIFT:
SERUM NEOPTERIN AS AN IMMUNOLOGICAL MARKER OF DISEASE ACTIVITY IN INFLAMMATORY DISEASES.

1. Neopterine waarden geven een beter beeld dan het bepalen van de bezinking (BSE) als parameter voor de activiteit van een ziektebeeld bij infectie ziekten daar de waarde in het serum snel daalt en normaliseert in navolging van een behandeling.

2. Achtereenvolgende bepalingen van serum neopterine waarden bij een patiënt kunnen nuttig zijn voor het bepalen van het beleid bij de behandeling van ontstekingsziekten.

3. Om de ziekteactiviteit bij een patiënt met sarcoidose te vervolgen is de bepaling van de serum neopterine waarde beter dan de bepaling van het Angiotensin Converting Enzyme (ACE) in het serum.

4. De hypothese dat cel gemedieërde immuniteit (CMI) een rol speelt in Erythema Nodosum Leprosum (ENL) wordt gesteund door de verhoogde serum neopterine waarde.

5. De waarde van neopterine in het serum bij patiënten met borderline lepra kan nuttig zijn voor het differentiëren tussen enerzijds, onbehandelde patiënten en patiënten met een recidief, en anderzijds patiënten met een Reversal Reaction (RR).

6. Tolerantie betekent niet zich willoos laten overtroeven door andersdenkenden, maar wel samen met andersdenkenden de grens definiëren.

7. De ene cultuur is niet beter dan de andere cultuur. Ze is alleen anders.
De meerwaarde van een multiculturele samenleving is gelegen in de herkenning en de erkenning van het anders zijn.

8. De inzet van welgestelden voor het verrichten van verpleegkundige en andere zorg taken, in de geest van de uitspraken van Mgr. M. Muskens, zal leiden tot een hogere waardering van de beroepen van verpleegkundige en verzorgende.

9. Ondanks verbeterde diagnostiek in de bestrijding van lepra staat menselijke zorg (verlening) centraal.

10. Man must not play God.

F.F.V. Hamerlinck, september 1999.

Cover illustration: " Kokobè"
Painted by René Tosari, an artist from Suriname who lives and works in Amsterdam-Zuidoost. He studied at the 'Academie voor beeldende kunst' in Paramaribo and Rotterdam. He has had exhibitions all over the world.

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" Rap, uit mijnen weg en
uit mijn zunne, dat ik zie;
houdt op en laat mij werken,
of ik strale u!" zei de bie.

Guido Gezelle, 13 juni 1882
gedicht: wat hangt gij daar te praten.

Voor Brigitte,
met dank aan mijn ouders.

CONTENTS

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AIMS OF THE STUDIES

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The accumulated knowledge about the organization and function of the human immune system contributes to a better understanding of the pathogenesis of many diverse disorders and is opening new avenues for therapeutic regimens. In this thesis one component of the immune system, neopterin, has been studied to determine the status of the immune system in different inflammatory diseases and to evaluate the use of it in monitoring therapeutic efficacy.

In **chapter 1** an up-to-date review is presented of the literature focused on the immunological and physiological properties of neopterin. Neopterin was discovered in bee larvae, in worker bees and in royal jelly. The compound was termed 'neopterin' to denote that it might start a new (Greek, neo) epoch in pteridine research. Increased concentrations of neopterin were reported in patients with viral infections, suggesting that increased neopterin may originate from the immune response of patients to the infections. In vitro studies revealed that human monocytes/macrophages produce neopterin when stimulated by interferon-γ. Neopterin can easily be detected in serum and urine. The most important clinical applications for the determination of neopterin are prognostic indicator of malignant diseases, follow-up control of chronic infections, monitoring of immune-stimulatory therapy, differential diagnosis of acute viral and bacterial infections, prognostic indicator in HIV infections and early indications of complications in allograft recipients. In recent years new physiological functions of neopterin have been discovered such as inducing or enhancing cytotoxicity, inducing apoptosis and the role of a chain breaking antioxidant. Ample evidence has been presented that in psoriatic skin immune activation takes place leading to interferon-γ production by T lymphocytes.

In **chapter 2** untreated patients suffering from mild to severe psoriasis, immune activation was monitored by serum IL-2 receptor, soluble CD27 and neopterin levels.

In **chapter 3** of this thesis the serum neopterin concentration was evaluated as an additional marker for disease activity in the primary cutaneous T cell lymphomas: mycosis fungoides and Sézary syndrome. The diagnosis was made on clinical and histological criteria according to the classification of the European Organization for Research and Treatment of Cancer (EORTC). Results were compared with those of patients with psoriasis, atopic dermatitis and healthy controls.

Reactions, a common phenomenon among leprosy patients under treatment, require early detection and proper management to prevent serious nerve damage.

It is generally accepted that these reactional states are immunologically mediated, and as such, improve with treatment with immunomodulatory drugs such as corticosteroids. In **chapter 4** we studied the relationship between the occurrence of leprosy reactions and serum neopterin concentrations, as well as the influence of treatment with corticosteroids. We used banked sera obtained from leprosy patients before, during, and after reaction. We compared neopterin levels in single serum samples from leprosy patients on treatment with and without reaction, with untreated controls, and when available, serial samples from patients with and without reaction. Sarcoidosis is an inflammatory multiorgan disorder of unknown origin, characterized by the infiltration of T lymphocytes and mononuclear phagocytes and by the formation of noncaseating granulomas in the affected organs. So far, prognostic parameters predicting deterioration are missing in untreated sarcoidosis. Current concepts of the immuno-pathogenesis of the disease include local stimulation and replication of activated T lymphocytes and macrophages via a complex cytokine network

In **chapter 5** serum neopterin concentrations were compared with two other serum parameters, angiotensin converting enzyme and lysozyme to evaluate the value of this parameters before, during and after treatment of (sub)acute and chronic sarcoidosis. Serum neopterin, angiotensin converting enzyme and lysozyme concentrations were measured in untreated patients with pulmonary tuberculosis and compared to those in patients with sarcoidosis to determine the usefulness of these serum parameters in an another granulomatous condition.

In **chapter 6** patients with visceral leishmaniasis and cutaneous leishmaniasis were studied. The diagnosis was confirmed by demonstration of parasites in samples from skin, aspirates of lymph node, bone marrow or spleen. Serum neopterin levels were determined during a longitudinal study of a population of visceral leishmaniasis patients treated with sodium stibogluconate. The aim of the study was to investigate the value of the serum neopterin concentration as a marker of disease activity and as a parameter of the efficacy of the treatment. There is a need for a simple test for field use which allows early detection of an infection and a fast determination for an useful treatment. The extent and activity of infections with intracellular microorganisms e.g. *Mycobacterium tuberculosis, M. leprae* and *Leishmaniasis* correlate significantly with elevated neopterin levels.

In **chapter 7** a rapid and simple semi-quantitative dipstick assay for the determination of the serum neopterin concentration is described which may be valuable as a diagnostic tool and to monitor the effectiveness of therapy.

Chapter 1

NEOPTERIN: A REVIEW.

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ABSTRACT

Neopterin was discovered in bee larvae, in worker bees and in royal jelly. The compound was termed 'neopterin' to denote that it might start a new (Greek,neo) epoch in pteridine research. Increased concentrations of neopterin were reported in patients with viral infections, suggesting that increased neopterin may originate from the immune response of patients to the infections. In vitro studies revealed that human monocytes/macrophages produce neopterin when stimulated by interferon-γ. Neopterin can easily been detected in serum and urine. The most important clinical applications for the determination of neopterin are prognostic indicator of malignant diseases, follow-up control of chronic infections, monitoring of immune-stimulatory therapy, differential diagnosis of acute viral and bacterial infections, prognostic indicator in HIV infections and early indications of complications in allograft recipients. In recent years new physiological functions of neopterin have been discovered such as inducing or enhancing cytotocicity, inducing apoptosis and the role of a chain breaking antioxidant. This review will focus on the immunological and physiological properties of neopterin.

Keywords:
neopterin - biosynthesis - cell mediated immunity - immunological marker

INTRODUCTION

In 1889 Hopkins isolated a pigment from the wings of lepidoptera (1). This work was continued by Wieland and Schopf (2), who in 1936 named these pigments pteridines (3), a term which has its origin in the Greek word for wing, pteron. However, attempts to elucidate the structures of these compounds were unsuccessful until Purrmann (4,5,6) showed that three insect pigments, xanthopterin, isoxanthopterin and leucopterin, contain the bicyclic nitrogenous ring pyrazino-(2,3 -d)-pyrimidine. The bicyclic nitrogenous ring system pyrazino-(2,3 -d)-pyrimidine is now termed pteridine according to the international Union of Pure and Applied Chemistry. Neopterin was isolated from larvae of bee (7), from worker bees, and from royal jelly (8) in 1963. Originally, H. Rembold intended to term the new compound, 2-amino-4-hydroxy-(erythro-1',2',3'-trihydroxypropyl)—pteridine, "novapterin," to indicate that it was a new (from Latin, novum) molecule isolated from honey bees (Latin, Apis) and with a pterin structure. The compound finally was termed "neopterin" to denote that it might start a new (Greek, neo) epoch in pteridine research. In 1967, Sakurai and Goto isolated 25 mg of neopterin from 500 liters of human urine (9). Following the identification of a pteridine as the fluorescent component that was elevated in the urine of mice with Ehrlich ascites tumor, compared to healthy mice, the corresponding substance from human urine was isolated and characterized. It was found that the fluorescent component previously observed in urine of patients with malignant diseases was neopterin. Wachter and co-workers found elevated rates of neopterin excretion in a group of patients with various malignant disorders, as well as in patients with viral diseases (10). In 1981, it was suggested that neopterin originated from the immune response of the host directed against tumor cells or virally transformed cells (11). Further in vitro studies (12,13,14) revealed that human monocytes/macrophages produce neopterin when stimulated by interferon-γ. This lymphokine is released from activated T cells. Other cell types do not produce measurable amounts of neopterin following various stimuli. Therefore, neopterin production appears to be closely associated with activation of the cellular immune system. These in vitro experiments are consistent with the results of numerous clinical studies.

CHEMISTRY

Chemical reactivities indicate that both neopterin and its acid-oxidizable reduced forms (total neopterin) can be measured with sufficient accuracy. Urine and serum specimens, however, sometimes have to be shipped from the clinician or physician to the laboratory, and sometimes have to be stored for a longer time. In this case, the acid-oxidizable reduced forms of neopterin are converted to a variable extent into dihydroxanthopterin, xanthopterin, and pterin. Storage for a longer period at -20°C does not influence the neopterin concentration. Furthermore, a study using freshly collected and uniformly handled samples (15) demonstrates that the ratio of neat neopterin to total neopterin (neopterin + 7,8 dihydroneopterin) has a fairly constant value for both urine and serum.

BIOSYNTHESIS

Neopterin and its derivatives are synthesized in vivo from guanosine triphosphate (GTP) via GTP cyclohydrolase I (GTP-CH). The activity of GTP-CH can be greatly enhanced by interferon-γ (16,17). 7,8-dihydroneopterintriphosphate (NH2TP) is on the biosynthetic pathway of 5,6,7,8-tetrahydrobiopterin (BH4). BH4 represents the electron donor in the hydroxylation of phenylalanine to tyrosine in the liver and of tyrosine to L-dopa and tryptophan into 5-hydroxy-tryptophan in neuroendocrine tissue synthesizing cathecholamines or serotonin (18). In the above mentioned tissues and in lymphocytes the majority of NH2TP is

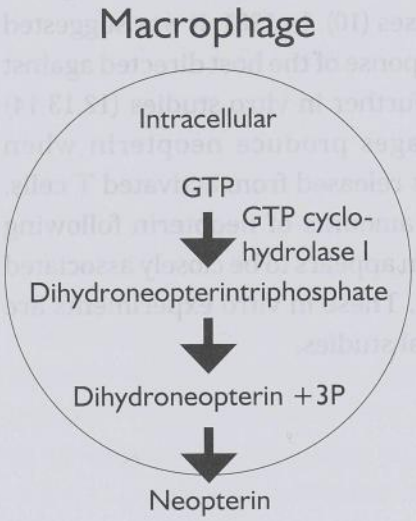

Fig. 1. Human monocytes/macrophages lack the enzyme 6-pyruvoyl- tetrahydropterin synthetase which converts NH2TP to 6-pyruvoyltetra-hydropterin (17). As a consequence, monocytes and macrophages instead of synthesising BH4 accumulate NH2TP which , after hydrolysis by phophatases, is excreted as dihydro-neopterin (NH2) or neopterin (19,20).

metabolised to BH4 (8,9). Human monocytes/macrophages lack the enzyme 6-pyruvoyl- tetrahydropterin synthetase which converts NH2TP to 6-pyruvoyltetra-hydropterin (17). As a consequence, monocytes and macrophages instead of synthesizing BH4 accumulate NH2TP which , after hydrolysis by phophatases, is excreted as dihydroneopterin (NH2) or neopterin (19,20). Fig. 1 On the basis of this biochemical in vitro evidence it has been concluded that increased neopterin biosynthesis during inflammatory disease is primarily derived from interferon-γ activated monocytes/macrophages. However, production of neopterin, a presumed primate homologue of nitric oxide in lower animals, was increased in THP-1 cells stimulated with interferon-γ and TNF-α. α-MSH significantly inhibited this production. The evidence indicates that an autocrine regulatory circuit based on α-MSH occurs in human monocyte/macrophages (21).

PHYSIOLOGICAL ROLE OF NEOPTERIN

Since the main physiological role of interferon-γ may be the induction of antibacterial, antiprotozoal, and antifungal activities of parasitized macrophages, it has been suggested that neopterin might act as an endogenous inhibitor of folate synthesis by intracellular pathogenic microorganisms (14). At slightly alkaline pH (pH 7.5) neopterin enhances hydrogen peroxide and chloramine-T activity. This is demonstrated by increase of signal intensity in aluminol assay and also by enhancement of toxicity towards bacteria. Thus, the macrophage derived substance neopterin is able both to enhance and to reduce cytotocicity dependent of the pH value and the oxidation state of neopterin, and it may have a pivotal role in modulation of macrophage mediated effector mechanism (22). Recent data implied a potential role of neopterin derivatives in oxygen free-radical-mediated processes, e.g. high concentrations of 7,8-dihydroneopterin were found to interfere with the oxidant-antioxidant balance, and may lead to apoptosis of human cells (23,24). In addition, 7,8-dihydroneopterin was found to be effective in the activation of redox-sensitive transcription factors and in the induction of HIV-1 gene expression . Neopterin and 7,8-dihydroneopterin are inducers of apoptosis which is not mediated by nitric oxide (25). In vitro 7,8-dihydroneopterin increases in a dose dependent manner, the lag time of low density lipoprotein oxidation mediated by Cu++ ions or peroxyl radical generator 2,2'-azobis (2 -amino propane) dihydrochloride (AAPH). 7,8-

dihydroneopterin also inhibits AAPH mediated oxidation of linoleate. The kinetic of the inhibition suggest that 7,8-dihydroneopterin is a potent chain breaking antioxidant which functions by scavenging lipid peroxyl radicals (26). Neopterin stimulates inducible nitric oxide synthetase (iNOS) gene expression in vascular smooth muscle cells in vitro. One possible explanation for the impact of neopterin on iNOS gene expression is that neopterin activates the translocation of NF-kappa B sub-units to the nucleus by modulating the intracellular redox state (27). The enhancing potency of the oxidized form of neopterin towards long-wave ultraviolet light (UV-A) induced cytotoxicity was examined in vitro using mouse melanoma (B-16) cells. The results suggest that elevation of the hydrogen peroxide-mediated cytotocicity by neopterin may be involved in its enhancing potency toward UVA-induced B-16 cell damage, and may also indicate the possible utility of the oxidized form of neopterin as an enhancer for UV-A irradiation treatment of tumors (28). Furthermore several pteridines - i.e. neopterin - influenced the intracellular calcium level in human monocytes. Whether this elevation of intracellular calcium level is caused by direct influence of neopterin on the calcium channels or by synergistically effects has to be investigated in the future (27). A summary of the main physiological properties of neopterin is demonstrated in Table 1.

Table 1. Main physiological properties of neopterin

- endogenous inhibitor of folate synthesis by intracellular pathogenic microorganisms
- enhance hydrogen peroxide and chloramine- T activity at pH 7.5
- enhance and reduce cytotocicity dependent of the pH value and the oxidation state
- modulation of macrophage mediated effector mechanism
- a role in oxygen free-radical -mediated processes e.g. apoptosis of human cells
- activation of redox-sensitive transcription factors
- induction of HIV-1 gene expression
- inducer of apoptosis which is not mediated by nitric oxide
- increasing the lag time of low density lipoprotein oxidation
- inhibits AAPH mediated oxidation of linoleate
- a potent chain breaking antioxidant
- stimulates inducible nitric oxide synthetase (iNOS) gene expression
- enhancing potency towards long-wave ultraviolet light (UV-A) induced cytotocicity
- influences the intracellular calcium level in human monocytes

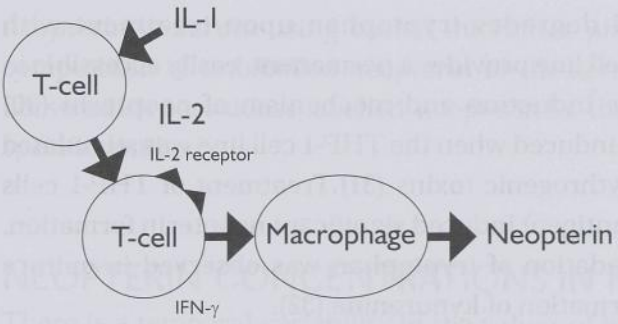

Fig. 2. Macrophages and lymphocytes modify the behaviour of each other in part, through the release of bioactive molecules such as interferon-g and interleukin-1. Interleukin-1 acts on T cells in two ways: it induces receptors for interleukin-2, which would then allow the T cell to respond to this T cell growth factor; it also stimulates interleukin-2 production. Interleukin-2 triggers T cells to secrete interferon-γ.

CELLULAR SOURCE AND INDUCTION SIGNAL

T Lymphocytes play a role in nearly all skin diseases in which defense mechanisms are involved, or where primary intrinsic aberrations in the immune system are operative. The lesional infiltrate usually consists of different subsets of T cells, but the dominant T cell subset in the dermis is almost always the CD4+ T cell along with an admixture of CD8+ cells. Macrophages and lymphocytes modify the behavior of each other in part, through the release of bioactive molecules such as interferon-γ and interleukin-1. Interleukin-1 acts on T cells in two ways: it induces receptors for interleukin-2, which would then allow the T cell to respond to this T cell growth factor; it also stimulates interleukin-2 production. Interleukin-2 triggers T cells to secrete interferon-γ. Fig. 2 Various in vitro and in vivo studies demonstrated that in inflammatory diseases stimulation of macrophages with T cell derived interferon-γ led to significant increase of GTP cyclohydrolase I activity and of neopterin concentration. Macrophages, when exposed to interferon-γ release large amounts of neopterin. Two different mechanisms underlay this phenomenon; first, directly interferon-γ stimulates the activity of the key enzyme cyclohydrolase I; second, human macrophages lack the activity of the 6-pyruvoyltetrahydropterin synthetase, the first enzyme after dihydroneopterin triphosphate. As a consequence, monocytes and macrophages instead of synthesizing BH4 accumulate NH2TP which, after hydrolysis by phosphatases, is excreted as dihydroneopterin NH2 or neopterin (29). The human myelomonocytic cell line

THP-1 forms neopterin and degrades tryptophan upon treatment with interferon-γ. Thus the THP-1 cell line provides a permanent, easily accessible in vitro system for studying the induction and mechanism of neopterin (30) Neopterin production was not induced when the THP-1 cell line was stimulated with streptococcal-derived erythrogenic toxins (31).Treatment of THP-1 cells with 90K (a tumor-associated antigen) induced significant neopterin formation. In parallel a significant degradation of tryptophan was observed in culture supernatants leading to the formation of kynurenine (32).

METHODS OF DETECTION

Fully oxidized neopterin can be measured by high-performance liquid chromatography (HPLC), (33) and radioimmunoassay (RIA) (34). Both methods show comparable results (14,15). An ELISA test is also commercially available. Samples may be stored refrigerated at 2 - 8 °C for up to 24 hours, or for up to 6 months frozen at -20 °C, protected from light. The upper limit of the normal range is approximately 10 nmol/L serum (= 2.5 ng/ml) (37). Measurement by HPLC. A method was developed for rapid separation and sensitive quantitation of urinary neat oxidized neopterin by reversed-phase HPLC on a 10-micrm octadecylsilica column. The analyses are eluted with 15 mmol/liter potassium phosphate buffer at pH 6.4 and at a flow rate of 0.8 ml/min. Urinary neopterin can be measured by fluorescence and related to creatinine determined by ultraviolet absorption in order to account for fluctuating concentrations of urine. The method has good performance characteristics and is easy to handle. The procedure was modified for routine laboratory automated analysis without any pretreatment except dilution of samples with aqueous potassium phosphate buffer using guard columns (35). Determination of neopterin in serum by HPLC is more difficult to perform than in urine due to the presence of protein and due to the about 200-fold lower concentration of neopterin. Also in cerebrospinal fluid, neopterin can be measured by reversed-phase high-performance liquid chromatography (36). Measurement by RIA. The particular advantage of RIA compared to HPLC is its suitability for large scale applications. A RIA kit is commercially available. The RIA is based on the competition of unlabelled neopterin of the serum samples or standards and radiolabelled neopterin for the binding sites of the neopterinspecific antibody. Measurement by ELISA. The ELISA test is a competitive enzyme immunoassay for the quantitative determination of

neopterin in serum using coated microtiter plates. The test is based on the competition of unlabelled neopterin of the serum samples or standards and horseradish peroxidase labelled neopterin for the binding sites of the neopterin specific antibody.

NEOPTERIN CONCENTRATIONS IN HEALTHY SUBJECTS

There is a temporal variability in the values of immunological parameters in a healthy population (38). Neopterin concentrations measured in serum by RIA and HPLC are consistent. Concentrations of neopterin in serum from 662 apparently healthy individuals (ages 1 to 97 years, median 22 years) were measured by RIA and the results statistically analyzed. Three age groups were identified as showing significantly different values for neopterin (Kruskal-Wallis test, $p<0.0001$) but there was no statistically significant sex dependence (Kruskal-Wallis, $p>0.05$). Subjects between ages 18 and 75 years showed no significant age dependence of the serum concentrations, but children (<18 years) and elderly subjects (>75 years) had significantly higher neopterin concentrations than did the middle group. The 95th percentiles was chosen as the upper normal limits (37). These results agreed well with data obtained for 1837 blood donors (ages 18-67 years), who had a mean neopterin concentration of 5.89 nmol/L, SD 1.78 nmol/L, and a upper 99% confidence limit of 10.5 nmol/L, estimated with an assumption of Gaussian distribution (39). In healthy pregnant woman there is a significant correlation between neopterin increase and tryptophan decrease as well as kynunerine increase and tryptophan decrease (40).

DISEASES ASSOCIATED WITH ELEVATED NEOPTERIN LEVELS

Increased concentrations of neopterin and dihydroneopterin are found in serum, cerebrospinal fluid (CSF) and urine taken from patients with a wide variety of malignant and non-malignant conditions in which the cell mediated immune system is activated (41,42). Neopterin concentrations in serum or urine seem of equal value for diagnostic application as long as renal function is normal (43). Neopterin concentrations may be significantly increased in a particular disease state compared to controls, serial measurement of neopterin concentrations in a particular patient may be useful in monitoring the course of a condition. As

neopterin release depends on activation of macrophages, it is associated with a variety of conditions involving activity of cell mediated immunity (CMI) (44). Measurement of neopterin levels is a useful early indicator of complications in allograft recipients. In patients who received allografts of tissue such as kidney, liver, pancreas and heart, immunological rejections are indicated by increasing neopterin levels in urine (45) or serum (46,47,48), earlier (mean 2 days) than using conventional diagnostic procedures. Similar changes in neopterin levels occur in patients after bone marrow transplantation (49). Urinary nitric oxide (NO) and neopterin were significantly increased in children breast fed by mothers with silicone breast implants (BFSI) compared with controls. There was a significant inverse relationship between urinary neopterin excretion and the severity of esophageal dysfunction (50). In general, acute viral infections such as hepatitis, rubella, several herpes infections (e.g. herpes simplex, Epstein-Barr, cytomegalovirus) are accompanied by high neopterin levels (45,49,51). Elevated neopterin levels also occur during acute phase of Kawasaki disease (52). Urinary neopterin excretion is increased in patients with both progressive and relapsing multiple sclerosis and therefore has potential as a surrogate marker of the inflammatory component of multiple sclerosis disease activity (53). In infections, high neopterin levels usually decrease when antibodies against the pathogen become detectable, for example in children after vaccination with live vaccines (51). The extent and activity of infections with intracellular bacterial infections (e.g. Mycobacterium tuberculosis, M. leprae, or parasites e.g. malarial parasites Plasmodium falciparum or P. vivax) correlated significantly with elevated neopterin levels (54,55). Highest neopterin levels are associated with poorer prognosis in patients with septicemia and in allograft recipients, during cytomegalovirus infection. High neopterin levels have also been found in CSF of patients with cerebral infections and in some cases of multiple sclerosis. Measurement of CSF neopterin concentration may be useful for differentiating between hereditary progressive dystonia/dopa-responsive dystonia (HPD/DRD) and early-onset parkinsonism with dystonia (EOP-D) (56). In the auto-aggressive diseases as rheumatoid arthritis (57,58), Crohn's disease (59), ulcerative colitis (60), autoimmune thyroiditis (61), systemic lupus erythematosus (62) and early onset of autoimmune diabetes mellitus (63), neopterin levels are highest in acute phase of the disease and correlate with the extent and activity of disease. In rheumatoid arthritis neopterin is also detectable in synovial fluid (64). A similar correlation exists in patients with sarcoidosis (65) and in children with coeliac

disease (66). In several malignant diseases, haematological tumors, gynecological tumors (67), tumors of the genitourinary tract in males (68), in pediatric cancer (69) and in lung tumors (70), high neopterin levels are significantly associated with poor prognosis. In patients with malignant melanoma, there is a remarkable increase of the excretion of neopterin, which depends on the extent of the involvement and by contrast to this, in patients with Hodgkin's disease there is a remarkable decrease of the excretion of biopterin also dependent on the extent of the involvement. In both types of tumor disease the ratio neopterin/biopterin is remarkably increased as compared to controls (71). Lymphocyte proliferative response to PHA is significantly diminished in cancer patients, and this depression appears to be partly linked to systemic inflammatory respons. Plasma interferon-γ and urinary neopterin was significantly increased in cancer patients, whereas interleukin-4 was undetectable (72). Neopterin, interleukin-10 and interleukin-6 mean concentrations were significantly higher in cancer patients than in controls. Mean values of both neopterin and interleukin-6 were significantly higher in metastatic patients than in those with locally limited disease. This suggest that macrophage- and TH2-mediated immunosuppression may occur independently in solid tumors and that it becomes more evident with disease progression (73). Several clinical studies have noted elevated neopterin levels in HIV infection (74,75,76,77). Increased neopterin concentrations are prevalent in asymptomatic HIV antibody seropositive individuals. A predisposition to seroconversion and progressive disease is linked to certain behavioral and immunological conditions, in addition to a high risk of exposure to HIV (78,79,80). An individual with pre-activated T cells and macrophages, once infected with marginal amounts of HIV, will be more effectively infected since replication of HIV may start immediately (81). In a rural African population, high neopterin levels were found in apparently healthy subjects. Many of these showed subclinical parasitic infections (82). These elevated neopterin levels were found with a similar frequency in both sexes. Progressive HIV infection is associated with a further increase in neopterin levels (76). Asymptomatic seropositive individuals and those with persistent generalized lymphadenopathy have similar neopterin levels. The correlation with the Walter Reed Staging Classification is highly significant (83). A significant inverse correlation exists between neopterin levels and CD4+/CD8+ T cell ratios and absolute CD4+ T cell numbers (84) in ARC and AIDS patients, which is weak in asymptomatic individuals. Increase in CD8+ T cells do not seem to be directly correlated with increased neopterin levels. It is interesting to note that

in the final stage of AIDS, extremely low numbers of CD4+ cells and of total lymphocytes can induce the release of extremely high neopterin concentrations. Particularly in HIV+ patients with current opportunistic infections β-2-microglobulin, neopterin and CD14+ monocytes expressing Fc γ RIII are increased, while CD4+ lymphocyte counts are reduced (85). Besides the role of neopterin as sensitive indicator of disease activity in HIV infection, neopterin derivatives are associated with the cascade of events that regulate the HIV production in infected individuals and augment progression to higher stages of HIV-associated disease(86). There are two further conditions known to lead to elevated neopterin levels. One condition is impaired renal function, which leads to elevated neopterin levels in serum but to normal neopterin/creatinine ratios in serum and urine. The other condition is atypical phenylketonuria (87). This rare metabolic defect is caused by dihydrobiopterin synthetase deficiency of 6-pyruvoyltetrahydropterin synthetase, an enzyme eliminating the triphosphate group from dihydroneopterin triphosphate. Patients are commonly detected by postnatal screening for concentrations of serum phenylalanine.

COMPARISON OF NEOPTERIN CONCENTRATIONS AND OTHER LABORATORY PARAMETERS.

Measurement of neopterin and total neopterins (neopterin + dihydroneopterin), are of almost equal potential for clinical diagnosis. However, when measuring total neopterins, which includes oxidation of 7,8-dihydroneopterin to neopterin, more strict requirements of sample collection and handling are necessary to avoid degradation of the 7,8-dihydro derivative (88). In comparison with the direct measurement of interferon-γ the determination of neopterin levels represents various advantages. Interferon-γ is subject to fast degradation and is able to bound to soluble or cell bound receptors so that the actual biological effective concentration indicates deviations to the obtained free measurable concentration. In connection with neopterin all these problems do not appear. In renal allografts, rejection episodes were associated by increasing neopterin levels even when interferon-γ could not be detected. It has been assumed that interferon-γ is produced in peripheral tissues but rarely enters the bloodstream. In contrast, neopterin enters the circulation due to its small size and chemical stability (89). In addition, the clinical value of neopterin levels was compared to

that of β-2 -microglobulin for monitoring the course of disease in 116 renal allograft recipients (90). The data of these studies indicated that elevations of neopterin levels clearly preceded those of serum creatinine, but β-2-microglobulin levels remained nearly constant 4 days prior to and 4 days after bolus steroid therapy. Neopterin levels provide an especially valuable picture concerning clinical activity in diseases which show rapid and acute changes in the severity of the disease. Therefore, neopterin estimations may under these circumstances be more useful than erythrocyte sedimentation rate (ESR) estimations, which has a long latency period (95). Simultaneous determination of serum neopterin and C-reactive protein (CRP) showed an increase for both markers in infections in bone marrow transplantation patients, whereas an increased neopterin in the absence of increasing CRP was characteristic for graft vs host disease (GVHD) (92).

NEOPTERIN LEVELS AFTER VACCINATION

Vaccination of children with a live measles-mump vaccine showed increased neopterin levels in all post-vaccination-courses, also in asymptomatic ones. The typical pattern of sharply increased levels was observed, with a peak at the time when viremia is known to be highest in wild-type measles infection (12 to 15 days after vaccination). Subsequently, neopterin levels rapidly declined and normalized. This decline coincided with the period in which specific antibodies become detectable. It is important to note that in none of these children clinical symptoms were apparent.

INFLUENCE OF IMMUNOTHERAPY ON NEOPTERIN LEVELS

Patients undergoing immunostimulatory treatment with interferon-a, interleukin-2 or tumor necrosis factor (93), show increased levels of neopterin, probably due to the induction of immunoregulatory cascades which stimulate release of interferon-γ. The opposite has been observed during immunosuppressive therapy: for example, neopterin levels decrease when graft rejection is successfully treated by cyclosporin A or corticosteroids (37,38). Excretion of urinary neopterin decreases immediately at the start of therapy with the immunosupressant cyclosporine in patients with mycosis fungoides (94). Increased neopterin levels

also followed withdrawal of cyclosporine A, since this drug inhibits secretion of interferon-g by T cells (27). A significant increase in plasma levels of neopterin was seen in patients with primary hypogammaglobulinaemia after one bolus injection (400 mg/kg) of intravenous immunoglobulin (IVIG) (95). A fast increase of neopterin values in sera of advanced cancer patients was seen during subcutaneous treatment with recombinant interleukin-2 (96). Mean serum neopterin levels were elevated during polyclonal antibody therapy (ATG) and monoclonal (OKT3) in patients following organ transplantation (97). Plasma concentrations of interferon-γ are increased in several inflammatory conditions. Administration of interferon-α (rhIFN-α2b; 5 x 10(6) U/m2) to eight healthy human subjects in a randomized controlled cross-over study showed significantly increase of neopterin 10 hours after administration. (98).

CONCLUSION

Human monocytes/macrophages produce neopterin when stimulated by interferon-γ released from activated T cells. Neopterin production appears to be closely associated with activation of the cellular immune system. High neopterin levels are found in different inflammatory diseases and certain malignancies and can easily be measured in serum and urine. Determination of the neopterin level in serum and urine from these patients have been demonstrated to be predictive for the course and progression of the disease and the response to therapy as its level rapidly declines and normalizes. Patients undergoing immunostimulatory treatment show increased levels of neopterin, probably due to the induction of immunoregulatory cascades which stimulate release of interferon-γ. The opposite has been observed during immunosuppressive therapy. In recent years new physiological functions of neopterin have been discovered such as inducing or enhancing cytotocicity, inducing apoptosis and the role of a chain breaking antioxidant. Measurement of neopterin concentration in serum, plasma, urine or cerebrospinal fluid is easily to perform and can provide particular information.

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Chapter 2

NEOPTERIN, IMMUNE ACTIVATION AND PSORIASIS

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Sir, Fuchs and co-workers (September 21, p. 759) reported their neopterin measurements (serum and urine) in seven psoriasis patients. They found elevated neopterin levels and significant correlation with the psoriasis area and severity index (PASI) (1). Our data do not support these findings. In nine untreated patients suffering from mild to severe psoriasis immune activation was monitored by serum IL-2 receptor, soluble CD27 and neopterin levels. Serum IL2-receptor and soluble CD27 levels were elevated in all patients, thus indicating T cell activation (2). However, the neopterin level was only significantly elevated (12.8 nmol/L) in one patient with mild psoriasis (PASI=6.6). Serum neopterin levels in all other 8 patients, including 3 cases of severe psoriasis were not elevated (< 10nmol/L). Moreover, no correlation (r = -0.05) was found between neopterin levels and PASI (range 2.9-15.2; mean 9.3) (unpublished results). Ample evidence has been presented that in psoriatic skin immune activation takes place thus leading to interferon (gamma) production by T lymphocytes (3). However, we feel that this is not always sufficient to induce detectable neopterin production and release by monocytes/macrophages in patients suffering from mild to severe psoriasis.

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Chapter 3

INCREASED SERUM NEOPTERIN LEVELS IN MYCOSIS FUNGOIDES AND SÉZARY SYNDROME

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SUMMARY

Neopterin (6-D-erythro-trihydroprolypteridine) is a low-molecular-weight compound derived from guanosine triphosphate. This molecule is synthesized by the macrophage stimulated by interferon-γ. An increased neopterin level is a good reflection of the activation of cellular immunity. It has been suggested that activated macrophages even promote tumor growth. In several malignant diseases, elevated levels of neopterin in urine and serum were observed. Serum neopterin concentration was measured by Radio-Immuno-Assay in patients with mycosis fungoides (n:10) and Sézary syndrome (n:4). Results were compared with those of patients with psoriasis (n:10), atopic dermatitis (n:10) and healthy controls (n:10). Neopterin levels were significantly elevated in patients with mycosis fungoides compared with patients with psoriasis vulgaris, atopic dermatitis and healthy controls ($P<0.05$). There was no significant difference between Sézary syndrome and psoriasis vulgaris, atopic dermatitis or healthy controls ($P>0.05$).These findings indicate that serum neopterin concentrations may be a marker of disease activity in mycosis fungoides.

Key words:
neopterin mycosis fungoides Sézary syndrome psoriasis vulgaris atopic dermatitis

INTRODUCTION

Neopterin is synthesized in vivo by macrophages from guanosine-triphosphate (GTP) via a series of reactions where the first isolable intermediate 7,8-dihydro-neopterin-triphosphate has been found. The enzyme GTP-cyclohydrolase-I (EC 3.5.4.16) - catalyzing this reaction - is regulated in a multitude of human and murine cells by cytokines, especially by interferon-γ. Human monocytes/ macrophages can be placed in an exceptional position as the activity of the constitutive enzymes is extremely low compared with the interferon-γ dependent GTP-cyclohydrolase-I. Consequently, in monocytes and macrophages instead of synthesizing biopterin, the intermediate 7,8-dihydroneopterintriphosphate is accumulated and after hydrolysis by ubiquitous phosphatases and oxidation it is excreted as 7,8-dihydroneopterin or neopterin in blood and urine (1). Evidence for elevated pteridine biosynthesis accompanied with increased neopterin levels can be observed in various diseases which are characterized by the stimulation of the cellular immune system. The latter are viral or bacterial infections (especially intracellular bacteria's) or parasites, autoimmune diseases, certain malignant tumors or allograft rejection (2,3).Thus, the level of neopterin measured in serum and other body fluids allows an inference on the in vivo-activation of the cellular immunity. In one publication elevated levels of neopterin in urine were found in psoriasis patients but not in a heterogeneous group of cutaneous T cell malignancies (4). Activated T lymphocytes have been shown to play a pivotal role in the expression of human immunodeficiency virus in cultures (5) and in patients (6). The purpose of this study is to evaluate the serum neopterin concentration as an additional marker for disease activity in the primary cutaneous T cell lymphomas, mycosis fungoides and Sézary syndrome.

PATIENTS AND METHODS

Patient population

Sera of eight patients with mycosis fungoides (MF) and four patients with the Sézary syndrome (SS) from the Department of Dermatology, University Hospital Utrecht, and two patients with mycosis fungoides from the Department of Dermatology, Academic Medical Centre Amsterdam, were involved in this study. The diagnosis was made on clinical and histological criteria according to the classification of the European Organization for Research and Treatment of

Cancer (EORTC) (7). In this study three patients with MF were included with stage 1a (MF confined to the skin with < 10% surface area involved), one patient with stage 1b (MF confined to the skin with > 10% surface area involved), four patients with stage 1c (MF confined to the skin with skin tumors), two patients with erythroderma (i. c. mycosis fungoides) and four patients with Sézary syndrome (Table 1).

Table 1. Serum neopterin levels in mycosis fungoides and Sézary syndrome

Pat.	m/f	age	stage	present status	serum neopterin level (nmol/L)
1	m	48	1a	PD	5.5
2	f	77	1a	PR	6.9
3	f	72	1a	PR	9.5
4	m	66	1b	CR	6.9
5	f	83	1c	D	13.5
6	m	83	1c	D	17.0
7	f	75	1c	D	27.4
8	m	63	1c	D	54.4
9	m	86	erythrod.	D	13.3
10	m	84	erythrod.	D	18.9
11	m	28	SS	D	5.7
12	m	71	SS	D	6.9
13	m	60	SS	D	9.8
14	f	72	SS	D	15.6

Staging classification according to the EORTC (10)

1: mycosis fungoides confined to the skin
a: limited plaques, papules or eczematous lesions
b: generalized plaques, papules or eczematous lesions
c: tumors
PD: progressive disease
PR: partial remission
CR: complete remission
D: died

Control subjects

Sera from 10 untreated patients with mild to severe chronic plaque psoriasis, 10 patients with untreated mild to severe atopic dermatitis and 10 healthy volunteers without skin diseases were used as control sera.

Laboratory investigation:

Neopterin levels in the sera of patients and controls were determined using a commercially available radioimmunoassay kit (Henning, Berlin, FRG). The upper limit of the normal range is approximately 10 nmol/L serum (= 2.5 ng/ml) (8).

RESULTS

Control groups.

Serum neopterin levels of ten patients with psoriasis vulgaris had a range of 3,8-6,8 nmol/L (mean 5,7 nmol/L) and in ten patients with atopic dermatitis the serum neopterin concentration had a range of 2.9-9.9 nmol/L (mean 6.4 nmol/L). In ten healthy volunteers the serum neopterin concentration had a range of 3,8-7,6 nmol/L (mean 5,4 nmol/L). (Table 2)

Table 2. Comparison of serum neopterin levels in nmol/L in patients with Mycosis Fungoides (MF), Sézary syndrome (SS), Healthy Controls (HC), Psoriasis Vulgaris (PV) and Atopic Dermatitis (AD)

Pat. Group	N	Range	Mean	SD	p *
MF	10	5.5 - 54.4	17.5	14.6	< 0,01
SS	4	7.1 - 15.6	9.5	4.3	ns >0,05
HC	10	3.8 - 7.6	5.4	1.2	-
PV	10	3.8 - 6.8	5.7	0.9	-
AD	10	2.9 - 9.9	6.4	2.4	-

* ANOVA - test

Patients.

In ten patients with mycosis fungoides, six males and four females aged between 48 and 86 years, the serum neopterin concentration had a range of 5,5-54,4 nmol/L (mean16,3 nmol/L). The three patients with stage 1a and the one patient with stage 1b had serum neopterin concentrations below the upper limit of the normal range, ranging from 5,5 - 9,5 nmol/L. All four patients with stage 1c and the two patients with erythroderma had serum neopterin values above the upper limit of the normal range, ranging from 13,5 - 54,4 nmol/L. (Table 1) One patient with stage 1c was followed during treatment with UV-A in combination with psoralens (PUVA). A serum sample was taken before therapy and eight weeks later during therapy. There was clinically amelioration of the disease and the serum neopterin level dropped from 17,0 nmol/L till 13,0 nmol/L. In four patients

with the Sézary syndrome, the serum neopterin concentration had a range of 5.7-15,6 nmol/L (mean 9,5 nmol/L) (Table 2).

Statistics.

With the One-way analysis of Variance (ANOVA) there was a significant difference between mycosis fungoides in comparison with psoriasis vulgaris, atopic dermatitis and healthy controls ($P<0.01$). There was not a significant difference between Sézary syndrome in comparison with psoriasis vulgaris, atopic dermatitis and healthy controls ($P>0.05$) (Table 2).

DISCUSSION

Serum neopterin levels in healthy controls were similar to those found in literature (8). In case of atopic dermatitis serum neopterin concentration was also below the upper limit of the normal range. This was already investigated before in vitro(9). Here the authors suggested that a possible dysregulation of interferon-γ may be related to increased IgE and IgG 4 production. In two studies pretreatment serum neopterin concentrations were not elevated in psoriasis patients and there was no correlation between improvement or deterioration of the psoriasis severity index score (PASI) and the serum neopterin levels (10,11). In contrast other workers showed that fully oxidized urine neopterin levels were significantly elevated in a psoriatic group but not in patients with mycosis fungoides (4). In this latter study there was a strong correlation between the urine neopterin concentration and the PASI score. Urine neopterin and its creatinine ratio were not significantly elevated in patients with mycosis fungoides and there was no correlation with the clinical stage of the disease. However, neopterin levels were elevated in patients with Sézary syndrome. In our study we did not find serum neopterin levels above the upper limit of normal range (< 10 nmol/L) in mild to severe psoriasis patients. An explanation for this contradictory result could be that urine neopterin concentration was determined as opposed to serum neopterin in our study. Although there is a close correlation between serum and urine neopterin levels, urine concentration is one thousand times greater than serum concentration. We found significant elevated levels of serum neopterin in mycosis fungoides as demonstrated in Table 1. This was due to high neopterin levels in stage 1c (n:4) and erythroderma (n:2) but not in

stage 1a (n:3) and 1b (n:1) which demonstrates a correlation between the stage of the disease and the serum neopterin concentration. These results suggest that in the case of a disseminated MF even higher serum neopterin concentrations could be expected. The reason why we did not find elevated neopterin levels in patients with Sézary syndrome (n:4), in contrast to the study mentioned above could be due to the exclusion criteria as e.g. the therapy before serum was taken for the determination of the neopterin concentration. In a previous report it was shown that a high urine neopterin concentration in one patient with mycosis fungoides treated with the immunosuppressant cyclosporine A fell after therapy with a markedly improvement of the clinical condition within eight weeks (12). We observed the same decline of the serum neopterin level with a clinical amelioration of the disease in one patient with mycosis fungoides stage 1c after eight weeks of treatment with PUVA therapy. High levels of serum neopterin in this study demonstrate the role of activated T lymphocytes in patients with mycosis fungoides and support the view that longitudinal studies could be of help in determining the use of neopterin concentrations during therapy, for the identification of relapses and the effect of the therapy. In case of Sézary syndrome , more patients should be evaluated before the start of chemotherapy.

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Chapter 4

SERUM NEOPTERIN AS A MARKER FOR REACTIONAL STATES IN LEPROSY

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SUMMARY

Reactions, a relatively common phenomenon among leprosy patients in treatment, require early detection and proper management to prevent serious sequelae. It is generally accepted that reactional states are immunologically mediated, and as such, usually improve with immunomodulatory treatments such as corticosteroids or thalidomide. Neopterin, a product of interferon-γ activated macrophages, is a marker for CMI activation and may be useful to detect reactional states in leprosy. Here, we compared neopterin levels in single serum samples from leprosy patients with and without reaction, with untreated controls, and when available, serial samples among patients with and without reaction. Levels in the single sample measurements, conducted in 22 patients with reversal reaction (RR; mean 14.5 nmol/L, SD 8.7) and 13 with erythema nodosum leprosum (ENL; mean 16.9 nmol/L, SD13.6), were significantly higher ($p = 0.02$ and $p = 0.001$, respectively) than levels in 26 untreated patients (mean 9.1 nmol/L, SD 7.3). Values above the upper limit of normal (10 nmol/L) were found in 7 of 26 untreated patients, 14 of the 22 RR patients ($p = 0.01$) and 10 of the 13 ENL patients ($p = 0.003$). Serial serum samples, obtained from 6 patients that developed reactions and 14 that remained free of reaction, indicated that RR or ENL paralleled a concomitant increase in the serum neopterin level. Neopterin levels generally declined upon corticosteroid therapy. Neopterin may be a useful marker for reactional states in leprosy by providing a laboratory parameter to assess onset, progression, response to therapy, and resolution.

INTRODUCTION

Leprosy is an unstable disease characterized by immunologically mediated reactional states such as reversal reaction (RR), occurring mainly in borderline lepromatous leprosy (BL), and erythema nodosum leprosum (ENL), occurring in lepromatous leprosy (LL) and BL [1,2]. ENL and RR may develop rapidly and can be associated with sequelae, most importantly permanent nerve damage (3). Although ENL is responsive to corticosteroids or thalidomide and RR improves with corticosteroids, there is no currently accepted laboratory parameter to identify patients at high risk of developing reactional states, their associated sequelae, or for monitoring the response to therapy (4,5). Neopterin, a pteridine compound synthesized from guanosine triphosphate (GTP) via GTP cyclohydrolase I in activated macrophages, is considered an early, specific, and sensitive marker of cell-mediated immune (CMI) activation (6). In vitro studies show that human monocytes (macrophages) produce neopterin when stimulated by interferon-γ from activated T cells (7,8,9). Other cell types do not produce measurable amounts of neopterin (10,11). Neopterin has been used to monitor CMI responses in acute allograft rejections, viral infections, intracellular infections, autoimmune diseases, and some malignancies (12). The value of obtaining neopterin levels to monitor for reactional states in leprosy patients is unclear. In East Africa, urine neopterin was increased in most LL as well as tuberculoid leprosy patients (13). However, there was no distinction between patients with and without reactions. Here, we studied the relationship between the occurrence of leprosy reactions and serum neopterin concentrations, as well as the influence of treatment with corticosteroids. To accomplish this, we used banked sera obtained from leprosy patients before, during, and after reaction.

MATERIAL AND METHODS

Patients and controls

The sera for this study were obtained from serum banks at Leonard Wood Memorial Center for Leprosy Research, Cebu, Philippines and the Department of Dermatology of the Academic Medical Center in Amsterdam, the Netherlands. The patients were classified according to the Ridley-Jopling scale (14). All clinical diagnoses were histologically confirmed. Multibacillary (MB) leprosy patients included all borderline and lepromatous patients with a bacterial index (BI) of

at least 2+ on the Ridley scale at any one site. Paucibacillary (PB) leprosy patients included indeterminate, tuberculoid (TT) and borderline tuberculoid (BT) with BIs < 2+ at any one site. Single serum samples were available from 26 untreated leprosy patients not in reaction, from 22 patients during RR before treatment and from 13 patients during ENL reaction before treatment. In addition, serial samples were obtained at fixed intervals during MDT from 14 patients who remained free of reactions within the period of follow-up (7 BL/LL; 7 TT/BT) and from 6 patients who developed a reaction. Of these 6, 4 patients developed RR (single episodes) and 2 patients developed multiple episodes of ENL (one with 4 episodes, the other 2 episodes). All 6 patients with reactional episodes received conventional doses of oral Prednison as therapy. Sera from 10 healthy Dutch subjects were used as controls.

Laboratory investigation

Serum neopterin levels of patients and controls were determined using a commercially available radioimmunoassay kit (Henning, Berlin, FRG). This radioimmunoassay is based on the competition of unlabelled neopterin of the serum samples or standards and radiolabelled neopterin for the binding sites of a neopterin-specific antibody. The radioactivity of the neopterin-antibody complex is reversibly proportional to the concentration of unlabelled neopterin in the sample. The upper limit of the normal range is approximately 10 nmol/L serum (= 2.5 ng/ml) (15).

Statistical Analysis.

The differences observed between the controls and the different patient groups were assessed by one-way analysis of variance (ANOVA).

RESULTS

The serum neopterin levels in each patient group are summarized in Table 1. The distribution of the serum neopterin concentrations are demonstrated in Fig. 1. Levels in patients with RR (mean 14.5 nmol/L, SD 8.7) and with ENL (mean 16.9 nmol/L, SD 13.6) were significantly higher ($p = 0.02$ and $p = 0.001$, respectively) than levels in untreated patients (mean 9.1 nmol/L, SD 7.3). No difference was found in neopterin levels between untreated PB and MB patients. Normal controls had a mean value of 5.4 nmol neopterin/L. Values above the upper limit of normal (10 nmol/L) were found in 7 of 26 untreated patients, 14 of

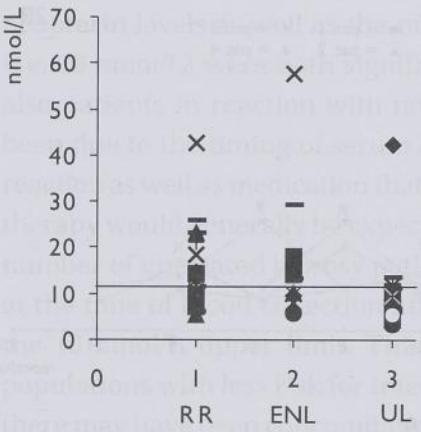


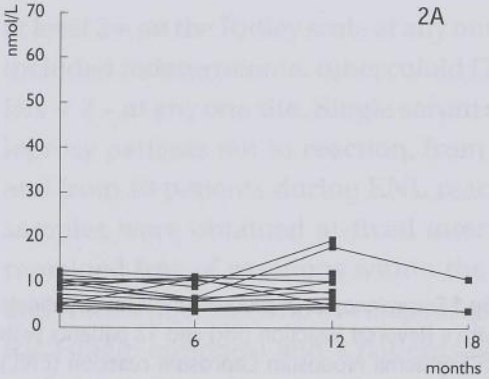
Fig.1 Serum neopterin values in nmol/L in 22 patients with a Reversal Reaction (RR) and 13 patients with an Erythema Nodosum Leprosum reaction (ENL) before treatment and 26 untreated leprosy patients (UL) without reaction.

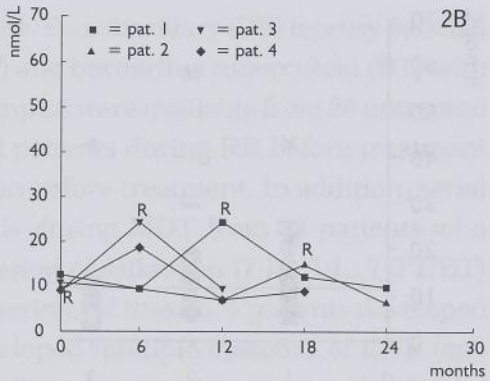
the 22 RR patients (p = 0.01) and 10 of the 13 ENL patients (p = 0.003). Figures 2A, 2B, and 2C show the neopterin levels in serially collected samples from patients with and without development of reactions. Figure 1A shows that the majority of leprosy patients who remained free of reaction during an 18 month follow up period (n = 14) showed little change in neopterin values. Notable exceptions occurred in 2 patients (18.4 and 19.7 nmol/L) at 12 months of follow up. Neither developed clinical evidence of reaction. Figure 2B shows neopterin levels in 4 patients that developed a single episode of RR. In 3 patients, elevated levels correlated with the development of RR. Upon Prednison administration, the levels in 3 patients dropped. In the 4th patient, who had already received Prednison for 3 months at the time of sampling, the levels were within normal limits (9.3 nmol/L). This patient received Prednison for 3 more months. Two months after treatment was stopped, the neopterin value rose to 18.5 nmol/L but by 4 months after treatment has stopped, the value was within normal limits and no further RR was noted. Figure 2C shows levels in 2 patients that developed ENL. One patient developed 4 episodes of ENL. In the period when this patient

Table 1

Patient Group	n	mean	SD	p
Untreated	26	9.1	7.3	—
RR	22	14.5	8.7	0.02*
ENL	13	16.9	13.6	0.001*

*Anova test





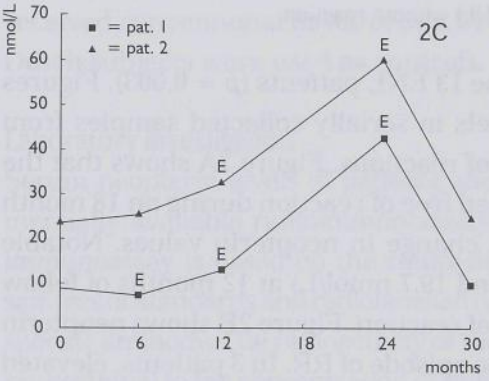


Fig. 2A
Serum neopterin concentrations during 18 months of follow up in 14 leprosy patients that remained free of reactions.

Fig. 2B
Serial serum neopterin levels in 4 leprosy patients who developed RR reactions (indicated by "R")

Fig. 2C
Serial serum neopterin levels in 2 leprosy patients who developed ENL reactions (indicated by "E")

did not receive Prednison therapy, i.e. from 10 days before the second episode of ENL until the day of blood collection at the third episode of ENL, neopterin levels in serum were above the limit of the normal range of 10 nmol/L. The second patient developed 2 episodes of ENL during follow-up and received Prednison therapy for 10 days in the period between the first and second episode of ENL.

DISCUSSION

It is generally accepted that reactional states in leprosy are strongly associated with CMI activation (16). Neopterin, a product of activated macrophages, may in that respect be expected to correlate with the development of reactional states (12). Here, we have shown that leprosy patients in reaction, either RR or ENL, have significantly elevated serum neopterin levels in comparison with patients not in reaction. A longitudinal assessment of 6 patients showed that an increase in neopterin generally paralleled the occurrence of reactions. Although the mean

neopterin levels as well as the number of patients above the limit of detection (i.e. 10 nmol/L) were both significantly higher in reactional states, there were also patients in reaction with normal neopterin serum levels. This may have been due to the timing of serum collection in relation to the progression of the reaction as well as medication that had already been administered. Corticosteroid therapy would generally be expected to reduce neopterin production (4). A small number of untreated leprosy patients without clinical signs of a reactional state at the time of blood collection had neopterin serum levels slightly higher than the 10 nmol/L upper limit. This upper limit is based on neopterin levels in populations with less risk for infections with microorganisms. In some patients, there may have been concomitant conditions that influenced the neopterin level. Longitudinal measurements in patients with and without reactions provided further insight into the value of neopterin levels. Neopterin levels clearly paralleled the occurrence of RR and ENL. However, neopterin levels in patients already receiving Prednison therapy were, not unexpectedly, relatively low. In the majority of patients not developing a reactional state, the neopterin levels did not increase above the upper limit of 10 nmol/L. As expected, this study showed that serum neopterin levels are generally increased during the development of reactional states and decline during immunosuppressive treatment. This is in agreement with our previous findings in which we demonstrated that neopterin levels were increased in RR and ENL compared to untreated TT/BT and BL/LL patients suggesting that CMI activation plays a large role in reactional states (17). Here we extended the study to confirm our initial results. However, elevated neopterin levels in a few patients not in reaction illustrate heterogeneity in neopterin production, emphasizing the importance of clinical observations. With this baseline data, we believe that a prospective study in which neopterin levels, alone or in combination with other immunologic markers, should be evaluated as a potential tool for the early detection of reactional states. Such a study might also be useful to determine whether neopterin levels discriminate between RR and a relapse, a distinction that is sometimes difficult.

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Chapter 5

SERUM NEOPTERIN IN SARCOIDOSIS; AN ADDITIONAL NON- SPECIFIC MARKER OF DISEASE ACTIVITY

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ABSTRACT

The purpose of the present study was to examine the additional usefulness of serum neopterin in the management of sarcoidosis. Neopterin is produced by the activated monocytes/macrophage lineage and is stimulated by interferon-γ. Other serum markers, such as lysozyme (LZM) and angiotensin-converting enzyme (ACE), are derived from macrophages/epithelioid cells in the characteristic granulomas of sarcoidosis. Serum LZM, ACE and neopterin were determined in 21 patients with biopsy-proven sarcoidosis who were classified into two groups: a (sub)acute group A (n=9) and a chronic group B (n=12). Sixteen patients, with or without therapy, were studied during a follow-up period (6-9 months). In addition, serum LZM, ACE and neopterin were measured in 7 patients with untreated pulmonary tuberculosis. Ten healthy control subjects presented the reference normal serum neopterin levels. Initial serum LZM, ACE and neopterin were increased in respectively 24%, 100% and 81% of all patients with sarcoidosis; as calculated, the mean values for serum ACE (102±28 U/L) and neopterin (23.1±22.2 nmol/L) were significantly ($p < 0.001$) elevated. LZM *versus* ACE was correlated ($r= 0.80$, $p < 0.001$), but neopterin was also correlated with LZM ($r= 0.86$, $p < 0.001$) and ACE ($r= 0.68$, $p < 0.001$). A difference was observed between the (sub)acute and chronic patients groups. In both groups only serum ACE and neopterin were significantly ($p < 0.001$) increased, but without any difference. In group A was no correlation between LZM and ACE *versus* neopterin. In group B, LZM *versus* neopterin ($r= 0.98$, $p < 0.001$)and ACE *versus* neopterin ($r= 0.87$, $p < 0.001$) were correlated. In group A and B serum LZM *versus* ACE correlation's were observed. In the follow-up study of patients with or without therapy the determination of serum neopterin indicated an early response to therapy or a relapse of the disease. There was no difference between the serum neopterin levels in untreated pulmonary tuberculosis and those in (sub)acute sarcoidosis. In conclusion, additional determined serum neopterin concentration proved to be an useful marker for disease activity in sarcoidosis, especially in the possibility of detection of monocytes/macrophage activation which may be an early phase in the ongoing granulomatous inflammation. The study indicates that serum neopterin, together with serum ACE, may be more or less helpful to distinguish the phase of the granulomatous inflammatory process in sarcoidosis.

Keywords: Serum neopterin, lysozyme, angiotensin-converting enzyme, sarcoidosis, tuberculosis

INTRODUCTION

Sarcoidosis is an inflammatory multiorgan disorder of unknown origin, characterised by the infiltration of T lymphocytes and mononuclear phagocytes and by the formation of noncaseating granulomas in the affected organs [1]. Current concepts of the immunopathogenesis of the disease include local stimulation and replication of activated T lymphocytes and macrophages via a complex cytokine network [2]. Previous studies have indicated that the release of neopterin, a metabolite of guanosine-triphosphate (GTP), reflects macrophage activation. Neopterin is produced by the monocytes/macrophages under the control of interferon-γ, one of the multiple cytokines derived from the activated T lymphocytes [3,4]. Its biological function in the immune and inflammatory reactions is unknown. The serum neopterin concentrations may be elevated in sarcoidosis and may reflect the intensity of the immune-mediated inflammatory process [5,6]. The incidental studies have focused on the relatively high sensitivity (72% and 62%, respectively) for serum neopterin in (in)active sarcoidosis. However, serum neopterin measurements are not routinely performed in the management of sarcoidosis. The low specificity (about 45%) of serum neopterin for sarcoidosis has limited its diagnostic value (7). Macrophages/epithelioid cell derived factors include mainly lysozyme (LZM) and angiotensin-converting enzyme (ACE). In clinical practice, the measurement of serum ACE is the most widely-used laboratory test in the management of sarcoidosis. Serum ACE levels varied widely and may be elevated in 40% to 90% of patients with sarcoidosis [7]. In one of our previous studies, 65% of all patients with newly diagnosed sarcoidosis had an initial increased serum ACE [8]. Serum ACE may be related to the presentation form and (or) different phases of the disease. The detection of an insertion/deletion polymorphism in the ACE gene also accounts for the variation in serum ACE levels [9,10]. Moreover, synthetic angiotensin-converting enzyme inhibitors may influence the test results. The aim of the present study has been to determine the usefulness of serum neopterin as a marker of monocytes/macrophage activation, and to investigate the relationship with the well-known activity markers LZM and ACE in the cascade of the granulomatous inflammation in sarcoidosis. Elevated urinary neopterin levels have been found in patients with tuberculosis and the authors indicated that the urinary neopterin levels were valuable for the treatment management of the disease [11]. In a small group of untreated patients with pulmonary

tuberculosis serum neopterin concentrations were measured and compared to those in patients with sarcoidosis in order to determine the usefulness of neopterin in another condition.

PATIENTS AND METHODS

Patients

The study concerned a group of twenty-one patients with sarcoidosis (14 women and 7 men) between 25 and 70 years of age (mean 42 years). All patients had a pulmonary manifestation of sarcoidosis attending the pulmonologic outpatients department. The diagnosis sarcoidosis was established by clinical evaluation (routine medical history and physical examination; laboratory tests, e. g. serum lysozyme and angiotensin-converting enzyme; chest radiography; pulmonary function tests) and was supported by tissue biopsy showing noncaseating epithelioid-cell granulomas. The histological diagnosis was obtained by peripheral lymph node biopsy (n=6), lymph node biopsy by mediastinoscopy (n=1), transbronchial lung biopsy (n=10), liver biopsy (n=1) and cutaneous biopsy (n=2) in 20 of the 21 patients. On the basis of the clinical evaluation all patients had an active disease. Twelve patients could be considered as chronic patients; the illness had persisted during 2-12 years (mean 7 years). According to the chest radiographic staging method the patients could be divided into four groups of pulmonary sarcoidosis: 6 patients with stage 1 (bilateral hilar and mediastinal lymph node enlargement without pulmonary abnormality), 8 patients with stage 2 (pulmonary involvement with bilateral hilar and mediastinal lymph node enlargement), 6 patients with stage 3 (pulmonary involvement without intrathoracic lymphadenopathy), and one patient had radiographic evidence of pulmonary fibrosis with contraction and distortion of lung architecture [12]. The clinical characteristics are summarised in table 1. No patient was taking corticosteroids at the time of the initial investigation. Sixteen patients had been followed on a three to six months schedule, with a maximum of fifteen months for one patient; 5 patients (patient no 12,16,17,18 and 19) had no therapy, 7 patients (patient no 1,4,5,6,10,15 and 21) were systemic treated with corticosteroids (Prednison) and in 4 patients (patient no 2,3,9 and 11) treatment with inhaled corticosteroids (budesonide) was initiated. One patient (no 4) with hypercalcemia and acute renal failure was additionally treated with

1000 mg methylprednisolone a day intravenously, for three consecutive days, which was repeated after two weeks; the maintenance dose of corticosteroids was 20 mg Prednison a day orally (table 1 and figure 6). During the follow-up of the 16 patients the disease (activity) was classified as improving, stable or deteriorating by clinical, radiographic and pulmonary function assessment. For comparison, serum samples of seven patients (4 women and 3 men; age between 24 and 62 years, mean 35 years) with untreated pulmonary tuberculosis were assayed for LZM, ACE and neopterin. The normal control group consisted of ten healthy subjects in whom only serum neopterin was measured.

Methods

All serum samples collected for routine LZM and ACE determinations were frozen and stored at -20° C in the dark until batchwise neopterin analysis was performed. No significant effect of storage, thawing and repeated freezing of the serum samples could be found. Serum neopterin was measured by a radioimmuno-assay using the commercial RIA kit from Henning (Berlin). The upper limit of the normal range is 10.0 nmol/L [13]. Serum LZM was measured with reagents from Instruchemie (Hilversum) and compared with two lysozyme standards. Lysozyme activity towards cell wall suspension of Micrococcus lysodeicticus was determined as described by Prockop and Davidson [14]. The normal range is 1.8-6.0 mg/L. Values exceeding the upper limit 6.0 mg/L were considered to be increased. Serum ACE was determined by the Bühlmann ACE kinetic test, using the commercial kit which was provided by DPC (Apeldoorn). Angiotensin-converting enzyme activity in serum was measured using L-hippuryl-L-histidyl-L-leucine as substrate [15]. In normal subjects the range of serum ACE is 18-55 U/L. A serum ACE value of 55 U/L was considered as the upper limit of the reference range.

Statistical analysis

Results are given as range and means ± SD. Intergroup differences were analysed by analysis of variance (ANOVA). A p-value of < 0.05 was considered to represent a significant difference.

RESULTS

Control subjects

In the ten healthy control subjects in whom serum neopterin was measured, all serum values were below 10.0 nmol/L (range 3.9-7.6 nmol/L; mean 5.3±1.2 nmol/L) corresponding with the values presented in the literature [12].

Patients with sarcoidosis

Table 1 shows the clinical characteristics of the 21 patients with sarcoidosis. Partly based on the criteria as formulated by the WASOG conference, the 12 chronic patients, as well as the 9 (sub)acute patients had an active disease [16]. In the 21patients with sarcoidosis serum LZM and ACE varied widely; the values were 2.2-18.8 mg/L (mean 5.8±3.8 mg/L) and 64-175 U/L (mean 102±28 U/L), respectively. Only the mean value for serum ACE was significantly ($p < 0.001$)

Pat. no.	Sex	Age	(Physical) examination	Radiographic stage	Biopsy	Presentation
1	F	31		2	TBB	1st visit
2	M	40	Salivary gland	2	TBB	1st visit
3	M	37		3	TBB	1st visit
4	M	65	Hypercalcemia, kidney	1	TBB, kidney	1st visit
5	M	40	Salivary gland, LN SC	2	LN SC	1st visit
6	F	65	Uveitis, skin	1	Skin	1st visit
7	F	25	Uveitis, EN	1	Skin	1st visit
8	F	36	Heerfordt syndrome	2	TBB	1st visit
9	M	40		3	TBB	1st visit
10	F	37			TBB	C; 2 yrs
11	F	33	Uveitis	2	TBB	C; 4 yrs
12	F	37	Uveitis, LN SC	1	Liver	C; 12 yrs
13	F	35	EN	1	-	C; 3 yrs
14	F	48	Salivary gland, LN SC	1	LN SC	C; 10 yrs
15	M	37	Skin, LN SC	2	LN SC, skin	C; 10 yrs
16	F	37	Skin	2	TBB, skin	C; 12 yrs
17	M	40	Uveitis, LN SC	3	LN SC	C; 10 yrs
18	F	38	Skin, LN SC	3	LN SC, skin	C; 6 yrs
19	F	37	LN SC	3	LN SC	C; 7 yrs
20	F	58		4	Med. scopy	C; 7 yrs
21	F	70	Uveitis, hypercalcemia	3	TBB	C; 3 yrs

F=female; M=male; EN=erythema nodosum; LN=lymph nodes; SC=supraclavicular; TBB= transbronchial lung biopsy; C=chronic disease Clinical characteristics of 21 patients with sarcoidosis.

Table 1. Clinical characteristics of 21 patients with sarcoidosis. Abbreviations: F=female; M=male; EN=erythema nodosum; LN= lymph nodes; SC=supraclavicular; TBB=transbronchial lung biopsy; C=chronic disease.

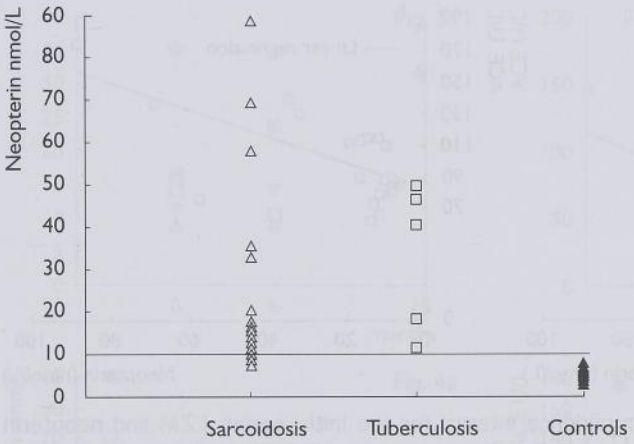

Fig. 1. Serum neopterin levels in 21 patients with sarcoidosis, 7 untreated patients with pulmonary tuberculosis and in 10 healthy control subjects. The horizontal line represents the normal upper limit of serum neopterin. Some values are not seen owing to overlying data points.

increased. Serum neopterin ranged from 6.6 to 69.2 nmol/L, mean 23.1 ± 22.2 nmol/L (figure 1). The mean serum neopterin was significantly ($p < 0.001$) elevated in comparison with the healthy controls. An initial increased serum LZM, ACE and neopterin could be determined in 24%, 100% and 81% of this series of patients. The serum levels LZM *versus* the serum levels neopterin correlated statistically significant ($r = 0.86$, $p < 0.001$) (figure 2a). There was also a significant positive correlation between serum ACE and neopterin concentrations ($r = 0.68$, $p < 0.001$) (figure 2b). Moreover, a significant correlation ($r = 0.80$, $p < 0.001$) was observed between the initial serum LZM and ACE levels. As shown in table 1 two categories of patients with sarcoidosis are presented: 9 (sub)acute patients were assigned to category A and 12 chronic patients to category B. No significant difference in age was found between the groups. By contrast, the chronic patients contained more females ($n = 10$). Separately, both categories of patients were also analysed. In category A the mean serum levels of LZM, ACE and neopterin were 4.1 ± 1.3 mg/L, 96 ± 17 U/L and 17.0 ± 15.8 nmol/L, respectively. Only the mean values for serum ACE and neopterin were significantly ($p < 0.001$) increased. No correlation's were found between the serum LZM and neopterin levels ($r = 0.10$, $p = 0.80$) and the serum ACE and neopterin levels ($r = -0.27$, $p = 0.48$). A significant correlation between the serum LZM and ACE levels was observed in the category A patients ($r = 0.66$, $p < 0.05$). In the patient group of category B the mean serum concentrations of LZM, ACE and neopterin were

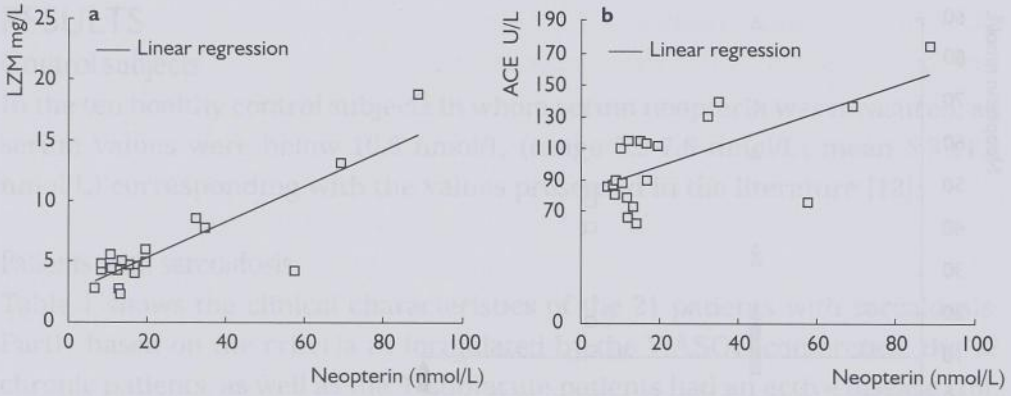

Fig. 2. Regression line with 95% confidence interval for the initial serum LZM and neopterin concentrations in 21 patients with sarcoidosis (a). Regression line with 95% confidence for the initial serum ACE and neopterin levels in the same group of patients (b). In both figures there are overlying data points.

15.3±32.7 mg/L, 106±34 U/L and 27.6±25.7 nmol/L, respectively. Serum ACE and neopterin were significantly ($p < 0.001$) elevated. In contrast with the results in the category A patients serum LZM and neopterin levels were significantly correlated ($r = 0.98$, $p < 0.001$). A correlation serum ACE *versus* serum neopterin was observed ($r = 0.87$, $p < 0.001$). We found also a correlation ($r = 0.75$, $p < 0.001$) between the serum LZM and ACE levels in this group of patients. There were no significant differences in serum LZM, ACE and neopterin levels between the two groups.

Follow-up of patients with sarcoidosis

Four chronic patients (no 12,16,17 and 19) who need no systemic treatment with corticosteroids could be followed during minimal 6 months. One patient (no 18) had only a follow-up at 15 months after the initial laboratory investigation. The individual serum concentrations of neopterin (a) and ACE (b) are given in figure 3. The disease was considered as stable in the patients no 16,17 and 19. The disease was deteriorating in the patients no 12 and 18, and were requiring treatment. The clinical deterioration was in concordance with the high serum neopterin concentrations (20.3-23.0 nmol/L and 34.8-31.0 nmol/L). Except for patient no 18 the serum ACE remained in the same elevated range. In seven patients (no 1,4,5,6,10,15and 21) treatment with Prednison was initiated (figure 4). In this small group of patients the initial serum neopterin levels varied widely (range

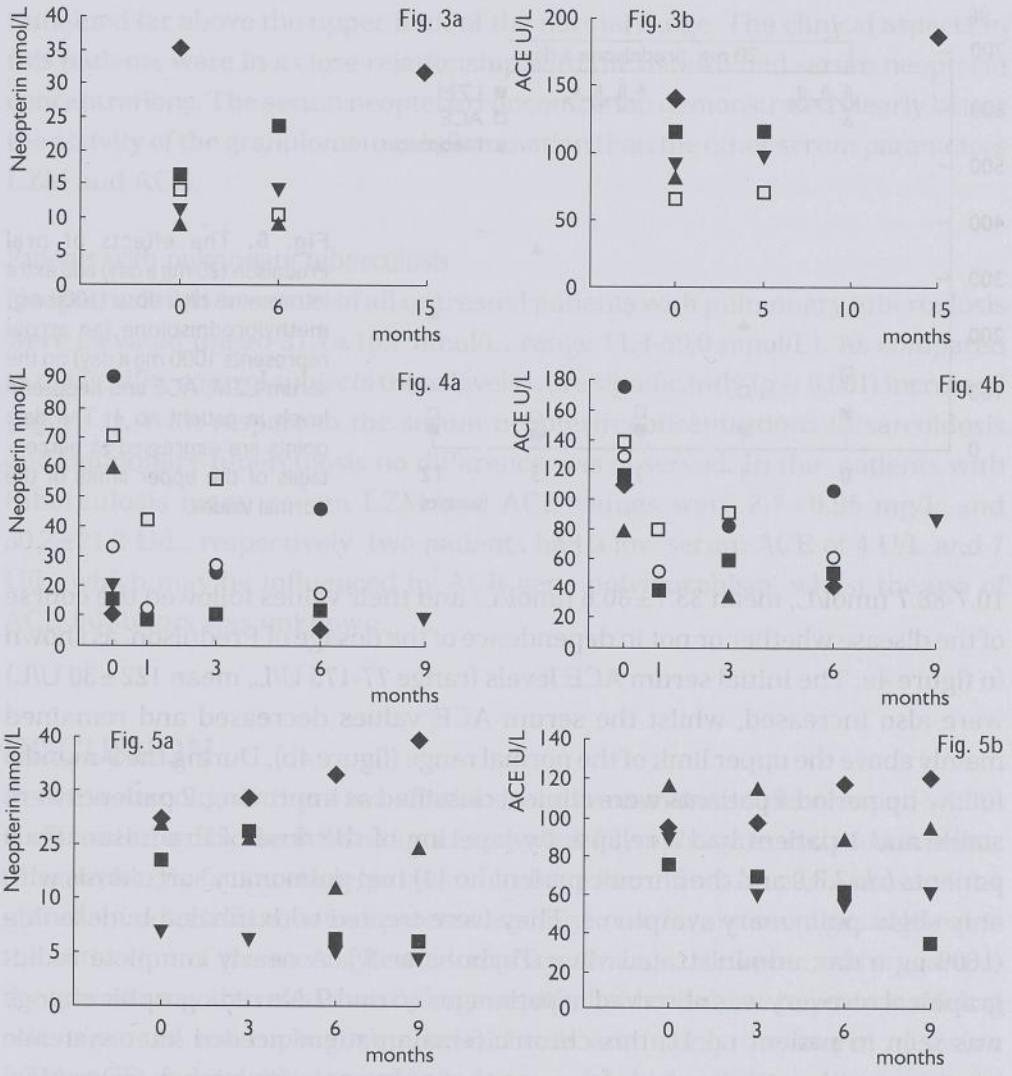

Fig. 3. Serum neopterin concentrations (a) and serum ACE levels (b) at the start of the study and during follow-up in 5 untreated patients (no 12,16,17,18 and 19) with chronic sarcoidosis. In both figures the overlying data points are limited.

Fig. 4. Follow-up of 7 patients (no 1,4,5,6,10,15 and 21) during treatment with different (maintenance) dosage of oral Prednison. a) The effect of the corticosteroids on serum neopterin. b) The effect of the corticosteroids on serum ACE. Some nearly same data points are not completely distinguished.

Fig. 5. Four patients (no 2,3,9 and 11) with pulmonary sarcoidosis could be treated with inhaled budesonide (1600 µg a day, administrated via a Turbuhaler ®). The figure shows the follow-up serum concentration of neopterin (a) and ACE (b).

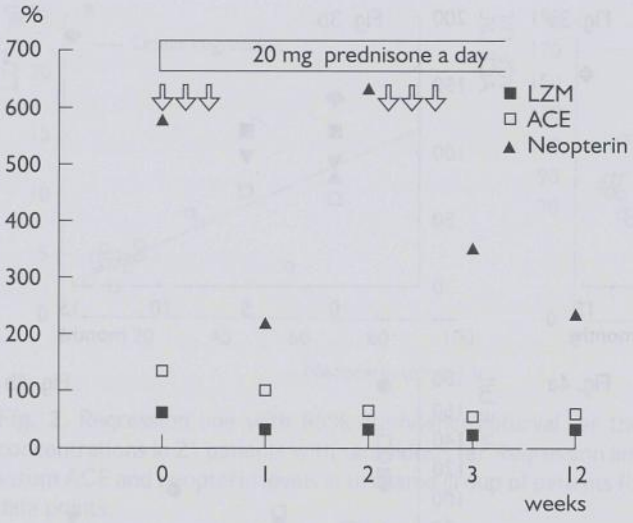

Fig. 6. The effects of oral Prednison (20 mg a day) and extra intravenous high dose (1000 mg) methylprednisolone (an arrow represents 1000 mg a day) on the serum LZM, ACE and neopterin levels in patient no 4. The data points are expressed as percentages of the upper limits of the normal values.

10.7-88.7 nmol/L, mean 33.7±30.6 nmol/L) and their values followed the course of the disease whether or not in dependence of the dosage of Prednison, as shown in figure 4a. The initial serum ACE levels (range 77-175 U/L, mean 122±30 U/L) were also increased, whilst the serum ACE values decreased and remained mainly above the upper limit of the normal range (figure 4b). During the 9-months follow-up period 4 patients were clinical classified as improving, 2 patients were stable and 1 patient had a relapse by tapering of the dose of Prednison. Four patients (no 2,3,9 and the chronic patient no 11) had pulmonary sarcoidosis with only slight pulmonary symptoms. They were treated with inhaled budesonide (1600 µg a day, administrated via a Turbuhaler ®). A nearly complete radiographical recovery was observed in patient no 2,3 and 9. No radiographic change was seen in patient no 11; this chronic female patient needed later systemic treatment with corticosteroids for reasons of pulmonary function deterioration. The serum neopterin and ACE concentrations of these patients during the follow-up period are presented in figure 5a and 5b, respectively. Figure 6 shows the results of the institution of corticosteroids pulse therapy because of hypercalcemia and acute renal failure. The renal failure was also histologically declared by the infiltration of granulomas in the kidney. Before treatment the serum neopterin concentration was very high, and it rapidly declined after the institution of corticosteroids, concomitantly with an improvement of the renal function. In contrast with serum LZM and ACE, in this patient serum neopterin

remained far above the upper limit of the normal range. The clinical aspects in this patients were in a close relationship with the determined serum neopterin concentrations. The serum neopterin concentration demonstrated clearly better the activity of the granulomatous inflammation than the other serum parameters LZM and ACE.

Patients with pulmonary tuberculosis

Neopterin levels in serum of all untreated patients with pulmonary tuberculosis were elevated (mean 31.1±16.7 nmol/L, range 11.4-50.0 nmol/L). As compared to the healthy control subjects these levels were significantly ($p < 0.001$) increased (figure 1). With respect to the serum neopterin concentrations in sarcoidosis and pulmonary tuberculosis no difference was observed. In the patients with tuberculosis mean serum LZM and ACE values were 2.7±0.95 mg/L and 30.2±21.2 U/L, respectively; two patients had a low serum ACE of 4 U/L and 7 U/L, which may be influenced by ACE gene polymorphism, whilst the use of ACE inhibitors was unknown.

DISCUSSION

In active sarcoidosis there is a continuous granulomatous inflammation which is characterised by the infiltration of activated T lymphocytes and mononuclear phagocytes. So far, no etiologic agent has been identified. An early phase in the granulomatous inflammatory process leading to the formation of granulomas is the activation of the monocytes/macrophage lineage. However, with regard to the granulomas activated macrophages/epithelioid cells have important clinical relevance; the macrophages/epithelioid cells are mainly the source of serum LZM, ACE and calcitriol, established as markers of activation in sarcoidosis. Serum ACE, reflecting the body granulomas load, is routinely used in the management of sarcoidosis. Current reports on serum ACE levels in patients with sarcoidosis have shown a sensitivity of 40 to 90% [7,8]. Serum ACE levels may be normal, especially in the (sub)acute disease. Differences in the reported sensitivity of serum ACE levels may also be related to the recently described insertion/deletion polymorphism in the ACE gene, the use of many different specific assays and the prescription of ACE inhibitors [7,9,10]. In the present study with selected patients groups the serum ACE level was elevated in all

patients. Our study demonstrates that additionally the serum neopterin levels, derived from the monocytes/macrophage lineage, were elevated in 81% of the patients. The levels correlated significantly with serum LZM and ACE concentrations, whilst serum LZM was increased in only 24% of this series of patients. The correlation's observed were not unexpected. The finding of a low sensitivity of serum LZM makes the measurement of LZM not meaningful in the clinical management of sarcoidosis. Subdivision of the patients in (sub)acute and chronic patients shows a difference in relationship between the determined serum LZM, ACE and neopterin levels. In both groups of patients the mean serum ACE and neopterin levels were in contrast with serum LZM significantly increased. Only in the group with chronic patients there was a correlation between the serum LZM and ACE levels *versus* the serum neopterin levels. There seems to be a closer relation between LZM and neopterin than between ACE and neopterin. The lack of correlation between either the serum LZM or serum ACE values *versus* the serum neopterin concentrations in the group of (sub)acute sarcoidosis patients may be due to more or less granulomas formation in the early phase of the ongoing inflammatory process. This observation is, so far as we know, not reported in the literature. The elevated mean serum ACE and neopterin concentrations were practically equal in both groups. Therefore, the described difference between the groups could not be explained by the extent of the disease, as shown in table 1. The present study indicates that neopterin gauges the activation of the monocytes/macrophage lineage and may be useful in an additional setting with angiotensin-converting enzyme of the macrophage/epithelioid cell lineage in the course of granulomas formation. As known, angiotensin-converting enzyme and neopterin are less specific and the measurement of these biochemical markers limits its diagnostic value. Nevertheless, the additional measurement of serum neopterin proved to be an useful marker for disease activity in sarcoidosis. The study suggests that the measurement of serum neopterin, together with serum ACE, may be more helpful in the management of patients with sarcoidosis, whether or not receiving therapy. The follow-up study confirms the usefulness of serum neopterin as a marker to monitor the disease activity. The marker seems to be superior to serum ACE. During the follow-up of patients with or without therapy the determination of serum neopterin may reflect in an earlier phase the response to therapy. The systemic administration of corticosteroids leads to a rapid reduction of the serum

neopterin level. The risk of relapse was sometimes shown by an elevation of serum neopterin, whilst serum ACE remained unchanged. In the small group of follow-up patients we found no prognostic value of neopterin with respect to the course of sarcoidosis. In patients with untreated pulmonary tuberculosis, a disease also characterised by the formation of granulomas, serum neopterin levels were significantly increased. No difference to sarcoidosis was distinguished. The measurement of serum neopterin is simple and inexpensive, and facilitates its use. Its additional use, together with the measurement of serum ACE, may be meaningful in the management of sarcoidosis.

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Chapter 6

SERUM NEOPTERIN CONCENTRATIONS DURING TREATMENT OF LEISHMANIASIS: USEFUL AS TEST OF CURE?

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SUMMARY

Neopterin, a product of interferon-γ activated macrophages was measured in sera from 28 patients: 12 patients with cutaneous leishmaniasis and 16 patients with visceral leishmaniasis, to determine the utility as a marker of disease activity and therapeutic efficacy. Patients originated from Kenya (n = 5) and from the Academic Medical Center, Amsterdam, the Netherlands (n = 23). In 7 patients follow up sera after treatment were available. Two patients at the time of diagnosis of visceral leishmaniasis were co-infected with HIV. The 12 patients with cutaneous leishmaniasis had serum neopterin levels below the upper limit of the normal range. All 16 patients with visceral leishmaniasis had elevated levels of serum neopterin before treatment. In 6 out of 7 patients with visceral leishmaniasis followed during treatment neopterin levels decreased to values below the upper limit of the normal range (10 nmol/L). Sequential measurements of serum neopterin levels may be useful for monitoring therapeutic efficacy in patients with visceral leishmaniasis.

INTRODUCTION

The leishmaniases are a group of diseases caused by *Leishmania* species. Depending on the species of parasite and the immune response of the host, manifestations comprise visceral leishmaniasis (VL) (kala-azar), cutaneous leishmaniasis (CL) (oriental sore), mucocutaneous leishmaniasis (ML) and post kala-azar dermal leishmaniasis (PKDL) [1]. Protozoa of the genus *Leishmania* are dimorphic obligate intracellular protozoan parasites that reside within mononuclear phagocytes in the mammalian host. The flagellated promastigote form of *Leishmania* spp. is delivered to the mammalian host by an infected sandfly. Promastigotes bind to specific receptors on macrophages and are internalized by receptor-mediated phagocytosis [2]. Opsonization with the third component of complement, C3, results in a striking enhancement of the binding and phagocytosis of *Leishmania major* promastigotes by macrophages [3,4]. After phagocytosis the parasites convert to nonflagellated amastigote forms that replicate and persist intracellularly. Since leishmania is an obligatory intracellular parasite, host defenses are dependent on T lymphocyte activity. T lymphocyte regions in the lymphoid organs become depleted and B cells proliferate with antibody production in response to infection. Proliferation of B lymphocytes, histiocytes and macrophages result in a lymphadenopathy and hepatosplenomegaly in VL. Cutaneous ulceration is characterized by mononuclear cell infiltrate, with Th1-type responses emerging after weeks of infection. Resolution of the infection depends on an increase in the number of leishmania-specific CD+ T cells of the Th1 subset with a granulomatous response with epithelioid and giant cells. Neopterin (6-D-erythro-trihydroprolypteridine) is a low-molecular-weight compound derived from guanosine triphosphate [5]. This molecule is synthesized by the macrophage stimulated by interferon-γ. An increased neopterin level is a good indicator of the activation of the cellular immunity. Many studies have reported elevated neopterin levels in various pathological situations: viral hepatitis [6], AIDS [7], intracellular bacteria [8] some autoimmune diseases [9], and graft rejection [10]. Serological tests for VL respond slowly to treatment and tests for the demonstration of parasites such as lymph node-, bone-marrow- or spleen aspiration are not practical for follow up. We were interested in the value of the serum neopterin as marker to monitor activity of disease and efficacy of treatment. We evaluated serum neopterin levels during a longitudinal study of a population of visceral and cutaneous leishmaniasis patients.

MATERIALS AND METHODS

Patients.

Twenty eight patients, 16 with visceral leishmaniasis and 12 with cutaneous leishmaniasis were studied (5 females, 23 males; age 4 - 85 years, Table 1). Twenty three patients were diagnosed and treated at the Academic Medical Centre, Amsterdam. Five Kenyan patients with visceral leishmaniasis were treated in the Kenyatta National Hospital and Infectious Disease Hospital, Nairobi. Two of the Dutch patients with visceral leishmaniasis were co-infected with HIV. In visceral leismaniasis, diagnosis was confirmed by demonstration of parasites in aspirates of lymph node, bone marrow or spleen. In cutaneous leishmaniasis the diagnosis was based on finding amastigotes free or in macrophages in Giemsa-stained smears or sections from biopsies or in culture from aspirates or biopsies. All patients were treated with sodium stibogluconate (Pentostam, Wellcome Co , London, UK) for 28 days, except one patient with cutaneous leishmaniasis acquired in the Amazone forest who was treated with pentamidine (Specia, Paris, France). Sera were available after treatment for variable periods of time from 7 of the 18 patients with visceral leishmaniasis. After completion of therapy of visceral leishmaniasis the efficacy of the treatment was assessed by parasitological examination of aspirates from spleen or bone marrow. In cutaneous leishmaniasis the completion of the therapy was based on clinical signs. Sera from ten healthy Dutch volunteers were used to confirm the normal range in comparison with the results found in literature.

Methods

Sera were stored at - 25° C until assays were performed. Neopterin levels in the sera of patients and controls were determined using a commercially available radioimmunoassay kit (Henning, Berlin, FRG). From all patients, except two, a direct agglutination test (DAT) was performed (Table 1). The DAT-test was performed as described previously with DAT - titers > 1:3200 were regarded as positive (11).

Statistical Analysis.

Comparisons were made using the Student t test. $P < 0.05$ was considered to represent a significant difference.

RESULTS

Serum neopterin levels in 10 healthy controls from the Netherlands had a mean value of 5.4 nmol/L with a range of 3.9 - 7.6 nmol/L which is in accordance with literature where values below 10 nmol/L are regarded negative [12]. All 12 patients with cutaneous leishmaniasis had normal serum neopterin values before treatment with a mean of 6.0 nmol/L (range of 5.6 - 7.6 nmol/L). Serum neopterin levels of 16 patients with visceral leishmaniasis were elevated before treatment with a mean value of 31.6 nmol/L (range 11.3 - 79.9 nmol/L). Two patients with visceral leishmaniasis who were co-infected with HIV had serum neopterin levels, respectively 30.0 nmol/L and 21.3 nmol/L before treatment. There was a significant difference between the neopterin concentrations in healthy controls and patients with visceral leishmaniasis ($P < 0.001$) before treatment. There was also a significant difference between the neopterin concentrations in patients with cutaneous leishmaniasis and patients with visceral leishmaniasis ($P < 0.001$) before treatment. Serum neopterin levels of seven patients with VL were followed after treatment for 6 - 12 months. After treatment 6 patients were parasitological cured. One patient did not respond to treatment and died 7 months after the start of treatment due to leishmaniasis (patient 1, Fig.1). At admission the

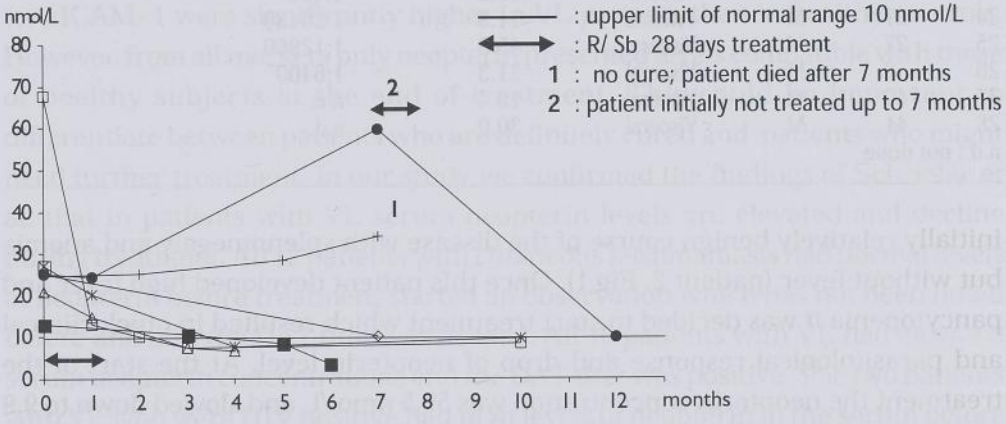

Fig.1 Serum neopterin concentrations before, during and after treatment with sodium stibogluconate in 7 patients with visceral leishmaniasis

neopterin concentrations varied from 13.1 - 67.6 nmol/L. In 6 patients serum neopterin levels normalized after treatment to normal values within 2-6 months (Fig.1). One patient was treated only 7 months after diagnosis because of the

Table 1. Serum neopterin concentration in nmol/L and the agglutination titer of a direct agglutination test (DAT) in 12 patients with cutaneous leishmaniasis and 16 patients with visceral leishmaniasis.

Pat.	Age	M / F	Cut./Visc. Leishmaniasis	Neopterin nmol/L	DAT titer
1	39	M	Cutaneous	5.6	< 1:100
2	26	M	Cutaneous	7.6	< 1:100
3	25	M	Cutaneous	6.5	1:400
4	23	M	Cutaneous	6.8	1:400
5	30	M	Cutaneous	7.2	< 1:100
6	31	M	Cutaneous	6.5	< 1:100
7	27	M	Cutaneous	6.4	< 1:100
8	37	M	Cutaneous	5.8	1:100
9	26	V	Cutaneous	6.5	< 1:100
10	20	M	Cutaneous	5.6	< 1:100
11	48	V	Cutaneous	6.1	< 1:100
12	23	M	Cutaneous	5.6	1:100
13	48	M	Visceral	79.9	1:104200
14	85	M	Visceral	25.2	1:104200
15	4	V	Visceral	59.5	1:25600
16	43	V	Visceral	32.8	1:104200
17	40	M	Visceral	67.6	1:184200
18	12	M	Visceral	21.0	1:12800000
19	8	M	Visceral	13.1	1:12800000
20	7	M	Visceral	27.0	1:12800000
21	10	M	Visceral	24.7	1:51200000
22	55	M	Visceral	30.7	1:102400
23	53	V	Visceral	19.2	1:400
24	27	M	Visceral	11.3	1:20480
25	27	M	Visceral	45.3	1:12800
26	59	M	Visceral	21.3	1:6400
27	40	M	Visceral	19.4	n.d.
28	41	M	Visceral	30.0	n.d.

n.d.: not done

initially relatively benign course of the disease with splenomegaly and anemia but without fever (patient 2, Fig.1). Once this patient developed high fever and pancytopenia it was decided to start treatment which resulted in quick clinical and parasitological response and drop of neopterin level. At the start of the treatment the neopterin concentration was 59.5 nmol/L and slowed down to 9.9 nmol/L after treatment. From the 14 patients with visceral leishmaniasis in whom the DAT test was performed only one patient had a negative DAT test (1:400). This patient had a lymph node leishmaniasis without splenomegaly and the neopterin concentration was 19.2 nmol/L (Table 1). In all 12 patients with cutaneous leishmaniasis the DAT titer was below 1:3200 and considered negative.

DISCUSSION

Histologically, cutaneous leishmaniasis lesions are characterized by an early influx of neutrophils, plasma cells and blood monocytes. In self-limiting forms of infection, the lesions mature slowly into granulomas consisting of infected cells surrounded by macrophages interspersed with lymphocytes and migrating neutrophils (13,14). T cells exert an anti-leishmania role by production of lymphokines such as TNF-α and interferon-γ [15,16]. In response to signals initiated by these activating factors, infected cells produce microbicidal molecules, such as reactive oxygen intermediates (ROI) and nitric oxide (NO) [17]. In the immune response to *Leishmaniae*, macrophages play an important role. Macrophages, when exposed to interferon-γ release large amounts of neopterin. Neopterin, in turn, is a good indicator of cell-mediated immunity [5]. Earlier studies examining markers in the circulation of patients with VL have focused predominantly on TNF-α and suggested that serum TNF-α concentrations are elevated during active VL and decline upon treatment and can be utilized to monitor disease activity [18,19]. Schriefer et al. in 1995 [20] evaluated different serum soluble markers for the evaluation of the treatment in human VL. The comparison of pre- and post-treatment concentrations showed that pretreatment serum levels of all markers (sCD4, sCD8 and neopterin) except for sICAM- 1 were significantly higher in VL patients than in healthy controls. However, from all markers only neopterin presented levels compatible with those of healthy subjects at the end of treatment. This could be important to differentiate between patients who are definitely cured and patients who might need further treatment. In our study we confirmed the findings of Schriefer et al. that in patients with VL serum neopterin levels are elevated and decline during treatment. All 12 patients with cutaneous leishmaniasis had normal levels of neopterin before treatment started an observation which has not been noted before and also the DAT-titer was normal. All 16 patients with VL had elevated serum neopterin concentrations and the DAT-titer was positive. The two patients with VL who were HIV positive had high levels of neopterin in the serum before treatment was started. Several clinical studies have noted elevated neopterin levels in HIV infection. Increased neopterin concentrations are prevalent in asymptotic HIV antibody seropositive individuals (21). As a consequence, follow-up of serum neopterin levels in patients with visceral leishmaniasis already infected with HIV will not give useful information on the efficacy of the treatment

of leishmaniasis. A predisposition to progressive HIV infection is linked to certain immunological conditions [7,22]. An individual with preactivated T cells and macrophages, once infected with marginal amounts of HIV, will be more effectively infected since replication of HIV may start immediately (23). Despite being clinically silent, subclinical visceral leishmaniasis could have deleterious effects on the outcome of HIV-1 infection. In fact, this disease could worsen the consequences of HIV-1 infection and increase the replication of the virus (24,25). Unfortunately there was no serum available before the *Leishmania* infection occurred nor after treatment. Therefore we could not evaluate if serum neopterin levels were already elevated before leishmania infection occurred or declined after treatment. Our study suggests that sequential measurements of serum neopterin concentrations during the treatment of VL can be useful for monitoring therapeutic efficacy in patients with visceral leishmaniasis but possibly not in HIV infection. In addition we demonstrated that the serum neopterin levels in cutaneous leishmaniasis were not elevated before treatment. Further study of the potential of neopterin as a marker of cure seems warranted.

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Chapter 7

A SIMPLE DIPSTICK FOR SEMI-QUANTITATIVE DETECTION OF NEOPTERIN IN SERA

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ABSTRACT

Neopterin, a low molecular weight pteridine produced by macrophages, is closely associated with activation of the cellular immune system. Neopterin biosynthesis during inflammatory disease is primarily derived from interferon-activated monocytes/macrophages. Neopterin concentrations may be significantly increased in a particular disease state compared to controls. A follow-up of serum neopterin concentrations during the course of an infectious disease could be useful to measure the activity of the disease and the influence of the treatment. We have developed a simple dipstick assay for the semi-quantitative detection of the neopterin concentration in serum of patients during the course of an infectious disease with a performance comparable with an ELISA, but which does not require specialised equipment.

Keywords: neopterin, dipstick, ELISA

INTRODUCTION

Neopterin, a low molecular weight pteridine produced by macrophages, is closely associated with activation of the cellular immune system. Neopterin biosynthesis during inflammatory disease is primarily derived from interferon-activated monocytes/macrophages (1). T lymphocytes play a role in nearly all skin diseases in which defence mechanisms are involved, or where primary intrinsic aberrations in the immune system are operative. Neopterin levels provide an especially valuable picture concerning clinical activity in diseases which show rapid and acute changes in severity of the disease. Therefore, neopterin estimations may under these circumstances be more useful than ESR (Erythrocyte Sedimentation Rate) estimation, which has a long latency period (2). Neopterin concentrations may be significantly increased in a particular disease state compared to controls, serial measurements of neopterin concentrations in a particular patient may be useful in monitoring the course of a condition. Patients undergoing immunostimulatory treatment showed increased levels of neopterin, probably due to the induction of immunoregulatory cascades which stimulate release of interferon-γ. The opposite has been observed during immunosuppressive therapy. Neopterin levels decrease under treatment with cyclosporine A or corticosteroids (3,4) The extent and activity of infections with intracellular bacterial infections e.g. tuberculosis, leprosy and leishmaniasis correlate significantly with elevated neopterin levels (5,6). Currently, neopterin levels in body fluids are determined with either RIA or ELISA. Both techniques require specific expertise and a specialised laboratory setting. Wider application of neopterin measurement, especially in developing countries, requires a simple test allowing early detection of an infection and a fast determination for a useful treatment. Here, we describe the development of a simple dipstick assay for the semi-quantatative determination of neopterin in serum and compare its results with that of ELISA.

MATERIALS AND METHODS

Sera.

The sera used in this study were obtained from the serum bank of the Department of Dermatology of the Academic Medical Centre, Amsterdam. The sera (n=18) were from untreated patients suffering from pulmonary tuberculosis (n=5),

cutaneous (n=1) and visceral leishmaniasis (n=4), pulmonary sarcoidosis, toxoplasmosis (n=1), and from 2 healthy persons.

ELISA Neopterin

Quantitative determination of neopterin in serum was performed using a competitive enzyme immunoassay (ELISA) (ELItest®, Brahms Diagnostica, Berlin, Germany) (7). In brief, in non- coated plates, 50 ml of neopterin standards (n=6) (human serum spiked with 2-250 nmol/l neopterin), controls (n=2)(mean values of 7.3 ± 1.4 nmol/l and 71.1 ± 16.1 nmol/l, respectively) and serum samples (n=18) were mixed with 150ml of neopterin conjugated with alkaline phosphatase. The mixtures were transferred to microtiter plates pre-coated with polyclonal anti-neopterin-antibodies and incubated in the dark for 2 hours. During this incubation, the neopterin of the patient samples competes with the neopterin enzyme conjugated for the binding sites of these antibodies forming an immune complex bound to the solid phase. This step was followed by intensive washing to ensure the removal of all unbound components. Enzyme reaction was allowed for 30 minutes after addition of 100 ml of 4-nitrophenylphosphate per well. The reaction was stopped adding 100 ml of sodium hydroxide 2N. The intensity of the colour was measured in optical densities (OD) at a wavelength of 405 nm. The OD value depends on the amount of enzyme bound in the wells at a constant reaction time and is inversely proportional to the neopterin concentration in the patient sample. Thus, the higher the neopterin concentration in the sample the lower the OD value. The results were calculated using a curve created by plotting the OD values against the 6 standard concentrations of neopterin on semi-logarithmic paper.

Neopterin Dipstick

The dipsticks were prepared essentially as described before (7). Nitro-cellulose strips were coated with polyclonal sheep anti-neopterin serum in dilution's of 1:10, 1:20 and 1:40. Sera (n=18), neopterin standards (n=6)(human serum spiked with 2-250 nmol/l neopterin) and control sera (n=2)(mean values of 7.3 ± 1.4 nmol/l and 71.1 ± 16.1 nmol/l, respectively), each 50 ml, were mixed with neopterin-alkaline phosphatase conjugate (150 ml) and the dipsticks were incubated with this mixture for 2 h in the dark at ambient temperature. Next, dipsticks were washed with running tap water and incubated with the precipitating substrate Fast Blue BB (Sigma, St.Louis, USA) for 10 min at

ambient temperature. At the end of the incubation period the dipsticks were rinsed with tap water and, after removal of excess of liquid with a paper towel, air-dried at ambient temperature. Results of the dipsticks from unknown samples were compared with those obtained with the neopterin standards and ranked from 1 to 6, where 1 corresponded best with the colour intensity of the lowest neopterin standard and 6 with the highest neopterin standard. The three different coating concentrations of anti-neopterin on the dipsticks facilitated the reading of the results.

Statistical evaluation.

The correlation between the ELISA and dipstick for neopterin concentration determination was determined by the Spearman Rank Correlation test. The inter-observer variation between two experimenters was determined by calculating kappa values with 95% confidence intervals (CIs). Kappa values express the agreement beyond chance. Generally, a kappa value of > 0.80 represents almost perfect agreement beyond chance . Values below 0.40 represent slight agreement and values between 0.40 and 0.80 represent fair to good agreement.

RESULTS

Figure 1 shows the results of the dipstick using 6 standards consisting of human sera spiked with known amounts of neopterin, and the 2 control sera. It clearly shows that the colour of the dipsticks is inversely related to the neopterin concentration in the sample. The colour of the band with the lowest antibody coating concentration is the first to become faint. The colour intensity of the dipstick with control serum nr.1 corresponded best with that of standard nr. 2 (6.4 nmol/l) and that of control serum nr.2 with nr. 5 (100 nmol/l). Table 1 shows the ELISA and dipstick results obtained with 18 sera from different patients and

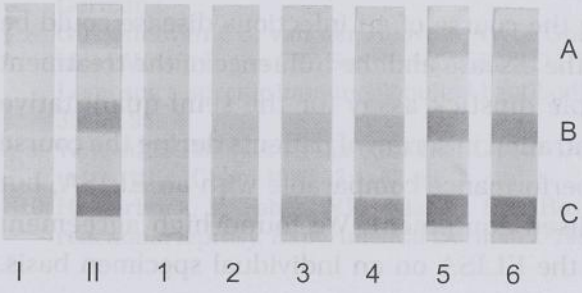

Fig.1. Dipstick results using sera spiked with 250 (stick 11), 100 (stick 2), 40 (stick 3), 16 (stick 4), 6.4 (stick 5) and 2 (stick 6) nmol/L of neopterin and two sera with an concentration of 71.1 (stick I) and 7.3 (stick II) nmol/L of neopterin. The sticks were coated with polyclonal sheep anti-neopterin serum in dilution's of 1:40 (band A), 1:20 (band B) and 1:10 (band C).

healthy persons. The dipstick results corresponded well with those of the ELISA: the Spearman's Rank correlation coefficient comparing ELISA and dipstick results was 0.77 (p=0.00006). As expected, the sera from the patient with cutaneous leishmaniasis and from the two healthy persons had low neopterin levels, as evidenced by both ELISA and dipstick results. Reading of the assay by two different experimenters resulted in an agreement of 80% (kappa value = 0.75).

DISCUSSION

Table 1. Comparison of ELISA and dipstick results.

Classification	ELISA (neopterin in nmol/l)	Dipstick (ranking 1-6)
Control 1 (7.3 nmol/l)	7.0	2
Control 2 (71.1 nmol/l)	76.3	5
Vis. Leishmaniasis nr. 1	70.1	4
Vis. Leishmaniasis nr. 2	63.8	5
Vis. Leishmaniasis nr. 3	80.0	6
Vis. Leishmaniasis nr. 4	26.7	3
Cut. Leishmaniasis nr. 5	6.5	1
Tuberculosis nr. 1	37.8	4
Tuberculosis nr. 2	63.1	4
Tuberculosis nr. 3	70.1	3
Tuberculosis nr. 4	25.9	2
Tuberculosis nr. 5	67.7	4
Sarcoidosis nr. 1	18.8	2
Sarcoidosis nr. 2	84.0	4
Sarcoidosis nr. 3	34.1	4
Sarcoidosis nr. 4	54.3	5
Sarcoidosis nr. 5	45.5	3
Toxoplasmosis	68.5	3
Healthy control nr. 1	3.4	1
Healthy control nr. 2	3.6	1

Although serum neopterin is not disease specific, an elevated serum neopterin concentration above the upper-limit of the normal range (10 nmol/L) gives an indication of the activation of cell-mediated immunity (9). A follow-up of serum neopterin concentrations during the course of an infectious disease could be useful to measure the activity of the disease and the influence of the treatment (10). We have developed a simple dipstick assay for the semi-quantitative detection of the neopterin concentration in serum of patients during the course of an infectious disease with a performance comparable with an ELISA, but which does not require specialised equipment. We found high agreement between the dipstick assay and the ELISA on an individual specimen basis.

Seropositivity rates obtained with the dipstick assay in different groups of patients with infectious diseases and controls did not significantly differ from the ELISA. Although the dipsticks results are assessed by the human eye as positive or negative, in contrast with ELISA results, which are determined by a spectrophotometer, the interpretation of the dipstick results was unequivocal, as illustrated by the high agreement between different test observers. In conclusion, the dipstick assay described here is an easy-to-perform method for the semi-quantitative measurement of serum neopterin concentrations in patients with inflammatory diseases. An internal control validates the performance of the assay. Due to its robustness and simplicity the dipstick assay seems to be highly suitable for application under field conditions and may prove to be suitable to quickly and easily assess elevated serum neopterin concen-trations in different inflammatory diseases.

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Chapter 8

SUMMARY AND CONCLUSIONS

Chapter 8

SUMMARY AND CONCLUSIONS

Chapter 1

Neopterin was isolated from larvae of bee (1), from worker bees, and from royal jelly (2) in 1963. Originally, H. Rembold intended to term the new compound, 2-amino-4-hydroxy-(erythro-1',2',3'-trihydroxypropyl)—pteridine, "novapterin," to indicate that it was a new (from Latin, novum) molecule isolated from honey bees (Latin, Apis) and with a pterin structure. The compound finally was termed "neopterin" to denote that it might start a new (Greek, neo) epoch in pteridine research. Following the identification of a pteridine as the fluorescent component that was elevated in the urine of mice with Ehrlich ascites tumor, compared to healthy mice, the corresponding substance from human urine was isolated and characterized. It was found that the fluorescent component previously observed in urine of patients with malignant diseases was neopterin. Wachter and co-workers found elevated rates of neopterin excretion in a group of patients with various malignant disorders, as well as in patients with viral diseases (3). In vitro studies (4,5,6) revealed that human monocytes / macrophages produce neopterin when stimulated by interferon-γ. This lymphokine is released from activated T cells. Other cell types do not produce measurable amounts of neopterin following various stimuli. Therefore, neopterin production appears to be closely associated with activation of the cellular immune system. These in vitro experiments are consistent with the results of numerous clinical studies. High neopterin levels are found in different inflammatory diseases and certain malignancies and can be measured in serum and urine. Determination of the neopterin level in serum and urine from these patients have been demonstrated to be predictive for the course and progression of the disease and the response to therapy as its level rapidly declines and normalizes (7). Patients undergoing immunostimulatory treatment show increased levels of neopterin, probably due to the induction of immunoregulatory cascades which stimulate release of interferon-γ. The opposite has been observed during immunosuppressive therapy. In recent years new physiological functions of neopterin have been discovered such as inducing or enhancing cytotocicity, inducing apoptosis and the role of a chain breaking antioxidant (8).

Chapter 2

Ample evidence has been presented that in psoriatic skin immune activation takes place thus leading to interferon gamma production by T lymphocytes (9). Fuchs and co-workers reported results (serum and urine) in seven psoriasis

patients (10). They found elevated neopterin levels and significant correlation with the psoriasis area and severity index (PASI). Our data do not support these findings. We hypothesize that in a local skin lesion the production and release of neopterin by monocytes/macrophages in patients suffering from mild to severe psoriasis is not always sufficient to induce detectable neopterin production.

Chapter 3

It has been suggested that activated macrophages even promote tumor growth. In several malignant diseases, elevated levels of neopterin in urine and serum were observed (11). We measured by Radio-Immuno-Assay in patients with mycosis fungoides and Sézary syndrome. Results were compared with those of patients with psoriasis, atopic dermatitis and healthy controls. Neopterin levels were significantly elevated in patients with mycosis fungoides compared with patients with psoriasis vulgaris, atopic dermatitis and healthy controls ($P<0.05$). There was no significant difference between Sézary syndrome and psoriasis vulgaris, atopic dermatitis or healthy controls ($P>0.05$). High levels of serum neopterin in this study demonstrate the presence of activated T lymphocytes in patients with mycosis fungoides and support the view that longitudinal studies could be of help in determining the utility of neopterin concentrations during therapy, for the identification of relapses and the effect of the therapy. In case of Sézary syndrome , more patients should be evaluated.

Chapter 4

Neopterin, a product of interferon-γ activated macrophages, is a marker for CMI activation and may be useful to detect reactional states in leprosy. Here, we compared neopterin levels in single serum samples from leprosy patients with and without reaction, with controls, and when available, serial samples among patients with and without reaction. Levels in the single sample measurements, conducted in patients with reversal reaction (RR) and with erythema nodosum leprosum (ENL) were significant higher than levels in untreated patients. Serial serum samples, obtained from patients that developed reactions and that remained free of reaction, indicated that RR or ENL paralleled a concomitant increase in the serum neopterin level. Neopterin levels generally declined upon corticosteroid therapy. Neopterin may be a useful marker for reactional states in leprosy by providing a laboratory parameter to assess onset, progression,

response to therapy, and resolution. Longitudinal measurements in patients with and without reactions provided further insight into the value of neopterin levels. Neopterin levels clearly paralleled the occurrence of RR and ENL. However, neopterin levels in patients already receiving Prednison therapy were, not unexpectedly, relatively low. In patients not developing a reactional state, the neopterin levels did not increase above the upper limit of 10 nmol/L. This study showed that serum neopterin levels are generally increased during the development of reactional states and decline during immunosuppressive treatment. However, elevated neopterin levels in a few patients not in reaction illustrate heterogeneity in neopterin production, emphasizing the importance of clinical observations. With these baseline data, we believe that a prospective study in which neopterin levels, alone or in combination with other immunologic markers, should be evaluated as a potential tool for the early detection of reactional states. Such a study might also be useful to determine whether neopterin levels discriminate between RR and a relapse, a distinction that is sometimes difficult.

Chapter 5

Sarcoidosis is an inflammatory multiorgan disorder of unknown origin, characterized by the infiltration of T lymphocytes and mononuclear phagocytes and by the formation of noncaseating granulomas in the affected organs (12). So far, prognostic parameters predicting deterioration are missing in untreated pulmonary sarcoidosis. Assessment of serum neopterin clearly demonstrated higher levels in patients with untreated acute form of pulmonary sarcoidosis than in normal subjects. Measurement of serum neopterin, can take a place in diagnostic strategies for dealing with pulmonary sarcoidosis because it generally reflects the presence or absence of a granulomatous process (13). In patients with pulmonary sarcoidosis and pulmonary tuberculosis we compared the serum neopterin level with two other biological markers: serum angiotensin-converting enzyme (ACE) and lysozyme (LZM). We found a clear difference with the three serum markers between acute and chronic sarcoidosis and determination of serum neopterin and ACE levels could be of help in differentiating between the two disease states. Elevated serum ACE levels were found in acute as wel as in chronic pulmonary sarcoidosis. Elevated serum neopterin levels were only found in the acute state of the disease. Serum neopterin concentration seems to be the most important marker in the follow up during treatment of patients with chronic

sarcoidosis or to prevent a relapse. There were no significant elevated serum levels found in the acute nor in the chronic form of pulmonary sarcoidosis for LZM. As we compared our results found in pulmonary sarcoidosis with pulmonary tuberculosis the same high concentrations for serum neopterin were found in the acute form of tuberculosis, demonstrating the activity of the macrophage in both disease states.

Chapter 6

The leishmaniases are a group of diseases caused by *Leishmania* species. Since leishmania is an obligatory intracellular parasite, host defenses are dependent on T lymphocyte activity. T cells exert an anti-leishmania role by production of lymphokines such as TNF-α and interferon-γ (14,15). In response to signals initiated by these activating factors, infected cells produce microbicidal molecules, such as reactive oxygen intermediates (ROI) and nitric oxide (NO) (16). In the immune response to *Leishmaniae*, macrophages play an important role. Macrophages, when exposed to interferon-γ release large amounts of neopterin. Neopterin, in turn, is a good indicator of cell-mediated immunity (17). Serological tests for VL respond slowly to treatment and tests for the demonstration of parasites such as lymph node-, bone-marrow- or spleen aspiration are not practical for follow up. All patients with cutaneous leishmaniasis had normal levels of neopterin before treatment; this has not been observed before, and also the DAT-titer was normal. All patients with VL had elevated serum neopterin concentrations and the DAT-titer was positive. In patients with visceral leishmaniasis followed during treatment neopterin levels decreased to values below the upper limit of the normal range (10 nmol/L). Our study suggests that sequential measurements of serum neopterin concentrations during the treatment of VL can be useful for monitoring therapeutic efficacy in patients with visceral leishmaniasis but possibly not in HIV infection. Further study of the potential of neopterin as a marker of cure seems warranted.

Chapter 7

Neopterin levels provide an especially valuable picture concerning clinical activity in diseases which show rapid and acute changes in severity of the disease. The extent and activity of infections with intracellular bacterial infections e.g. tuberculosis, leprosy and leishmaniasis correlate significantly with elevated

neopterin levels (18,19). Although serum neopterin is not disease specific, an elevated serum neopterin concentration above the upper-limit of the normal range (10 nmol/L) gives an indication of the activation of cell mediated immunity (20). A follow-up of serum neopterin concentrations during the course of an infectious disease could be useful to measure the activity of the disease and the influence of the treatment (21). The dipstick assay described here is an easy-to-perform method for the semi-quantitative measurement of serum neopterin concentrations in patients with inflammatory diseases. An internal control validates the performance of the assay. Due to its robustness and simplicity the dipstick assay seems to be highly suitable for application under field conditions

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Chapter 9

SAMENVATTING EN CONCLUSIES

Hoofdstuk 1

Neopterine is voor het eerst in 1963 geïsoleerd uit bijenlarven (1), uit bijen en uit honing (2). Oorspronkelijk wilde H. Rembold deze nieuwe stof, 2-amino4-hydroxy-(erythro-1',2',3'-trihydroxypropyl)-pteridine, novapterin noemen, om aan te geven dat het een nieuw (latijn: novum) molecule was geïsoleerd uit bijen honing (Latijn: Apis) en met een pterin structuur. De stof werd uiteindelijk "neopterin" genoemd om duidelijk te maken dat het mogelijk de start van een nieuw (Grieks: neo) tijdperk in pteridine onderzoek kon zijn.

Na de identificatie van een verhoogde concentratie van een pteridine als fluorescerende component in de urine van muizen met een Ehrlich ascites tumor in vergelijking met gezonde muizen, werd de overeenkomstige substantie geïsoleerd en gekarakteriseerd uit de urine van mensen. Hierbij werd vastgesteld dat de fluorescerende component welke vroeger geobserveerd werd in de urine van patiënten met een melanoom, neopterine was. Wachter en medewerkers vonden een verhoogde concentratie neopterine uitscheiding in de urine zowel bij groepen patiënten met verschillende kwaadaardige afwijkingen als bij patiënten met virale aandoeningen (3). In vitro studies (4,5,6) lieten zien dat menselijke monocyten/macrofagen neopterine produceren na stimulering met interferon-γ. Deze lymfokine word geproduceerd door geactiveerde T cellen. Andere cellen kunnen geen meetbare concentraties neopterine produceren na stimulering. Daarom lijkt neopterine produktie nauw samen te gaan met de activering van het cellulaire immuun systeem. Deze in vitro experimenten komen overeen met verschillende klinische studies. Hoge neopterine concentraties worden gevonden bij verschillende inflammatoire ziekten en bepaalde maligniteiten en kunnen gemeten worden in serum en urine. Bepaling van de neopterine concentratie in serum en urine van deze patiënten kan aanwijzingen geven over het verloop van de ziekte en het resultaat van de behandeling daar de concentratie snel daalt en normaliseert (7). Patiënten met een immuno-stimulerende behandeling laten een verhoogde concentratie neopterine zien, waarschijnlijk door de inductie van een immune regulerend systeem die het vrij komen van interferon-γ stimuleert. De laatste jaren zijn nieuwe fysiologische functies van neopterine ontdekt zoals het induceren en versterken van de cytotoxiciteit , het induceren van apoptosis en de rol van antioxidant (8).

Hoofdstuk 2

Herhaaldelijk is aangetoond dat in de huid van psoriasis patiënten immuun activatie plaats vindt en bijgevolg leidt tot interferon-γ produktie door T lymfocyten (9). Fuchs en medewerkers hebben de resultaten beschreven van serum en urine bij zeven patiënten met psoriasis (10). Zij vonden verhoogde neopterine spiegels en een significante correlatie met de ernst van de psoriasis uitgedrukt in de "psoriasis area and severity index" (PASI). Onze resultaten kunnen deze bevindingen niet bevestigen. Wij stelden vast dat de produktie en uitscheiding van neopterine door monocyten/macrofagen bij patiënten met een milde tot ernstige psoriasis niet altijd voldoende is om te kunnen meten.

Hoofdstuk 3

Geactiveerde macrofagen zouden tumor groei kunnen stimuleren. Bij verschillende kwaadaardige aandoeningen zijn verhoogde neopterin concentraties in serum en urine vastgesteld (11). Wij hebben door middel van Radio-Immuno-Assay bij patiënten met mycosis fungoides en Sézary syndroom neopterine gemeten. De resultaten werden vergeleken met patiënten met psoriasis, atopisch eczeem en gezonde personen. De neopterine concentraties waren significant verhoogd bij patiënten met mycosis fungoides in vergelijking met patiënten met psoriasis vulgaris, atopisch eczeem en gezonde controles ($P<0.05$). Er was geen significant verschil tussen patiënten met een Sézary syndroom en psoriasis vulgaris, atopisch eczeem en gezonde personen ($P>0.05$). Hoge waarden voor serum neopterine concentraties in deze studie wijzen op de aanwezigheid van geactiveerde T lymfocyten bij patiënten met mycosis fungoides en bevestigen de mening dat langdurige studies hulpvol zouden kunnen zijn in het bepalen van het nut van de neopterine concentratie gedurende de behandeling, voor het vaststellen van een relaps en het effect van de therapie. In het geval van Sézary syndroom zouden meer patiënten moeten geëvalueerd worden.

Hoofdstuk 4

Neopterine, geproduceerd door interferon-γ gestimuleerde macrofagen, is een marker voor CMI activiteit en kan hulpvol zijn om lepra in reactie vast te stellen. Wij hebben neopterine waarden in eenmalige serum monsters van lepra patiënten met en zonder reactie, vergeleken met gezonde controles en zo mogelijk opeen volgende serum monsters van patiënten met en zonder reactie over een langere tijd. Eenmalige serum neopterine waarden van patiënten met

een reversal reaction (RR) en patiënten met een erythema nodosum leprosum (ENL) waren significant hoger dan bij onbehandelde patiënten. Neopterine waarden van opeen volgende serum monsters van patiënten met en zonder reactie over lagere tijd genomen lieten zien dat bij RR en ENL de waarde van de neopterine concentratie overeen kwam met het voorkomen van een reactie. De neopterine concentraties daalden over het algemeen na behandeling met corticosteroïden. De neopterine waarde in het serum van patiënten met lepra in reactie kan een nuttige laboratorium parameter zijn om de aanvang, de progressie, invloed van de therapie en genezing vast te stellen. Bij patiënten die reeds Prednison kregen waren de serum neopterine waarden zoals verwacht relatief laag. Bij patiënten niet in reactie waren de serum neopterine waarden onder de 10 nmol/L. Deze studie laat zien dat serum neopterine waarden over het algemeen stijgen gedurende de ontwikkeling van een reactie en dalen tijdens immunosupressieve therapie. Enkele patiënten niet in reactie lieten eveneens een verhoging zien van de neopterine produktie wat het belang van het klinisch beeld benadrukt. Deze bevindingen laten zien dat een prospectieve studie, waarbij serum neopterine waarden vergeleken worden met andere immunologische markers nuttig kan zijn voor het vroeg vaststellen van een reactie. Een dergelijke studie zou eveneens hulpvol kunnen zijn om het verschil vast te stellen tussen een RR reactie en een relaps, een onderscheidt dat soms moeilijk te maken is.

Hoofdstuk 5

Sarcoidose is een inflammatoire multiorgaan ziekte met een onbekende ethiologie en gekarakteriseerd door infiltratie van T lymfocyten mononucleaire fagocyten en door de vorming van granulomen in de aangedane organen (12). Tot zo ver zijn er geen parameters bekend die verergering van het ziektebeeld bij long sarcoidose kunnen voorspellen. Bepaling van serum neopterine laat zien dat er bij onbehandelde acute sarcoidose hogere concentraties neopterine voorkomen dan bij gezonde personen. Het meten van de serum neopterine concentratie kan van diagnostische waarde zijn bij long sarcoidose daar dit over het algemeen de aanwezigheid of afwezigheid van een granulomateus proces weergeeft (13). Wij hebben bij patiënten met long sarcoidose en long tuberculose de serum neopterine waarde vergeleken met twee andere biologische parameters: serum angiotensin-converting enzyme (ACE) en lysozyme (LZM). Wij vonden een duidelijk verschil met de drie parameters tussen acuut en chronische sarcoidose. Bepaling van neopterine en ACE kan van hulpvol zijn bij het

differentiëren tussen de twee ziektebeelden. Verhoogde serum ACE waarden werden gevonden zowel bij acute als chronische long sarcoidose. Verhoogde serum neopterine waarden werden alleen gevonden bij een acute vorm van sarcoidose. Serum neopterine concentratie lijkt de belangrijkste parameter bij het vervolgen van een patiënt met chronische sarcoidose tijdens behandeling. Eveneens kan het van belang zijn om een relaps te voorkomen. Er waren geen significant verhoogde serum waarden bij de acute of bij de chronische vorm van sacoidose voor LZM. Ook bij acute long tuberculose werden dezelfde hoge waarden voor neopterine gevonden als bij acute long sarcoidose wat wijst op activiteit van de macrofaag bij beide ziektebeelden.

Hoofdstuk 6

De leishmaniasis zijn een groep van ziekten veroorzaakt door Leishmania soorten. Daar leishmania een intracellulaire parasiet is, is de verdediging van de gastheer afhankelijk van T cel activiteit. T cellen vervullen een anti-leishmania rol bij de produktie van lymfokinen zoals TNF-α en interferon-γ (14,15). In antwoord op signalen welke geïnitieerd zijn door deze factoren zullen geïnfecteerde cellen moleculen produceren die micro-organismen doden, zoals het reactieve zuurstof molecuul (ROI) en stikstofoxide (NO) (16). Tijdens de immuun respons tegen Leishmania spelen macrofagen een belangrijke rol. Macrofagen die blootgesteld zijn aan interferon-g produceren grote hoeveelheden neopterine. Neopterine op zijn beurt is een goede indicator van de cel gemedieerde immuniteit (17). Serologische testen voor VL reageren langzaam op een behandeling en testen voor het aantonen van parasieten zoals lymfklier-, beenmerg- of milt aspiraten zijn niet praktisch voor een vervolg studie. Alle patiënten met huid leishmaniasis hebben normale waarden voor neopterine voor behandeling; dit is nooit eerder aangetoond en ook de DAT-titer is normaal. Alle patiënten met VL hebben verhoogde serum neopterine waarden en de DAT - titers zijn eveneens positief. Bij patiënten met VL welke vervolgd worden tijdens behandeling dalen de neopterine concentraties tot waarden beneden de bovenste normaal grens (10 nmol/L). Deze studie laat zien dat het meten van serum neopterine concentraties tijdens de behandeling van VL nuttig kan zijn om het effect van de therapie te vervolgen. Dit is mogelijk niet het geval bij gelijktijdige HIV infectie. Uitgebreider studies om de waarde van neopterine te evalueren als een marker van behandeling zijn aan te bevelen.

Hoofdstuk 7

Serum neopterine waarden geven een nuttig beeld wat betreft de klinische activiteit van een ziekte bij snelle en acute veranderingen. De uitgebreidheid en activiteit van een infectie met intracellulaire bacteriën zoals tuberculose, lepra en leishmania komen significant overeen met de verhoogde neopterine concentraties in het serum (18,19). Alhoewel serum neopterine niet ziekte specifiek is geeft een verhoogde serum neopterine concentratie boven de bovengrens van de normaal waarde (10 nmol/L) een aanwijzing omtrent de activiteit van de cel gemedieerde immuniteit (20). Het vervolgen van serum neopterine concentraties gedurende het verloop van een infectie ziekte kan nuttig zijn om de activiteit van de ziekte en de invloed van de therapie te bepalen (21). De dipstick hier beschreven is een gemakkelijk uit te voeren test voor semi-quantitatief onderzoek van serum neopterine concentraties bij patiënten met inflammatoire aandoeningen. Een interne controle bevestigt de resultaten van de test. Door zijn degelijkheid en eenvoud lijkt deze dipstick test zeer geschikt voor toepassingen buiten een ziekenhuis.

Referenties: zie chapter 8 'Summary and Conclusions'

Chapter 10

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