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Urinary Neopterin Indicates Early Infection and Disease Progression: Model Studies with Simian and Human Immunodeficiency Viruses in Macaques

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Abstract

The value of urinary neopterin as a predictive marker for disease progression in SIV- and HIV-2-infected rhesus (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*) was assessed by comparing pre- with post-infection data. Before infection stable baselines for neopterin were observed in both species with significantly higher concentrations in cynomolgus macaques than in rhesus macaques. After infection of cynomolgus macaques with human immunodeficiency virus type 2 (HIV-2) neopterin concentrations exceeded 1.2 - 4.2 times preinfection values, whereas rhesus monkeys infected with simian immunodeficiency virus (SIV) yielded concentrations of more than 8 times above baseline. Increased neopterin concentrations always preceded seroconversion. No rise of neopterin was observed in cynomolgus macaques remaining seronegative after inoculation with HIV-2, whereas after inoculation with SIV neopterin was slightly elevated in rhesus monkeys despite remaining seronegative. In animals with high virus replication a pronounced increase of neopterin levels was followed by signs of immunodeficiency. Therefore like in HIV-1-infected man, in macaques infected with SIV or HIV-2 the urinary neopterin concentration is an early and reliably predictive marker for AIDS disease progression and reflects pathogenicity. This parameter can be easily assessed in study protocols for drug and vaccine tests in monkeys as in man.

Key words: Virus Isolation, Seroconversion, Animal Model, AIDS, Neopterin, SIV, HIV-2, macaques

Introduction

Several serological and immunological parameters have been investigated in the early and late stages of HIV-infection and have been assessed as predictive marker for disease progression. In the later stages of the infection, the most prominent change is a massive CD4⁺ cell depletion leading to reduced CD4⁺/CD8⁺ cell ratios (1). These parameters are inversely correlated to serum β_2 -microglobulin (B2M) (2) and neopterin (3-5) concentrations. HIV activity can be directly determined by measuring serum p24 antigen concentrations, but this antigen is not present in all patients and its reproducible quantitative determination is problematic (6). Also measurement of acid-dissociated p24 antigen has its limitations when compared to other predictive parameters in the course of HIV infection (7). To date direct quantification of HIV load by poly-

merase chain reaction is the method of choice for follow-up therapy success in HIV infected individuals (8).

For the very early phase of HIV-infection, the so-called primary HIV-infection (9), changes in unspecific immune activation markers such as serum neopterin and B2M (9) have been described (10,11). These parameters are probably interrelated. In contrast to most other markers of immune activation neopterin can also be measured in the urine (12) which represents a simple, non-invasive method to monitor alterations of the immune system even in the early stages of HIV-infection.

The experimental infection of macaques with human (13, 14) or simian immunodeficiency viruses (14) represent valuable animal models for AIDS research. In a preliminary study involving two rhesus monkeys, we could demonstrate that the increase in

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morning urinary neopterin concentration was a very early marker for the productive infection with simian immunodeficiency virus (SIV) isolate SIVmac251 (15). Similar results have been described for serum neopterin in SIV-infected (16) and for CSF in D-retrovirus-infected rhesus monkeys (17). In addition to SIVmac, macaques can be infected experimentally with other SIV isolates (14) and with HIV-2 (13, 14). In this study, urinary neopterin concentrations were systematically evaluated in 14 rhesus and 12 cynomolgus macaques infected with undiluted or serial dilution of either SIVagm, SIVmac or HIV-2ben.

Materials and Methods

14 rhesus monkeys (*Macaca mulatta*) of Chinese or Indian origin and 12 cynomolgus macaques (*Macaca fascicularis*) originating from the Philippines or Mauritius Island were experimentally infected. At the time of infection all monkeys were about 2 - 2.5 years old and seronegative for STLIV-1, SIV, and D-type retrovirus. Animal husbandry, blood and urine sampling and determination of neopterin have been described (15). Urine samples were stored at -20°C 2 - 4 hours after collection and sent at ambient temperature without further addition of preservatives once a week by regular mail to the investigating laboratory. Preinfection neopterin concentrations in morning urine samples were determined in relation to creatinine concentrations. Neopterin concentrations after infection were expressed as a percent of the individual mean levels before inoculation.

Virus isolates and their dilutions used to infect the two macaque species are shown in Tab. 1. All monkeys were inoculated intravenously either with cell-free virus taken directly from culture supernatant fluid or with dilutions of this material each in RPMI 1640 supplemented with 10 % fetal calf serum.

SIVagmTYO-1 had been passaged once in vivo prior to infection of two rhesus macaques. This virus was isolated from rhesus monkey #5264 ten weeks after infection (18). The original SIVmac251 (19) had also been passaged once in a rhesus monkey. This reisolate was designated SIVmac32H (20) and grown on C81-66 cells. Six rhesus monkeys were injected with one mL of a 10^{-2} dilution and two animals each with one mL of either a 10^{-4} or 10^{-6} dilution of this virus stock. HIV-2ben-infection of macaques has recently been described (21). Four monkeys from this earlier experiment, two rhesus and two cynomolgus macaques infected with an undiluted stock virus of HIV-2ben (stock 1), were also included in the present study. In order to determine the monkey infectious dose₅₀ (MID₅₀) of a different, newly prepared HIV-2ben

virus stock (stock 2) intended to be used as challenge virus for future vaccine experiments and grown on Molt-4 clone 8 cells, three cynomolgus monkeys each received 1 mL of a 10^{-4} or 10^{-5} dilution and two animals 1 mL of a 10^{-6} dilution. These dilutions were chosen according to the in vitro titer of 104.5 TCID₅₀/mL determined on MOLT-4 clone 8 cells. Two other cynomolgus macaques were inoculated with the undiluted stock virus derived from the biologically active molecular clone MK6 of HIV-2ben (22). SIVagmTYO-1 and SIVmac were isolated by cocultivation of Molt-4 clone 8 or C81-66 cells, respectively, with peripheral blood mononuclear cells (PBMC) of the inoculated monkeys. HIV-2ben reisolation and monitoring of seroconversion and immunological and clinical changes have been reported (21).

Grouped data were statistically evaluated with the Mann Whitney test. Neopterin concentrations were compared with preinfectious levels using Wilcoxon matched pairs signed rank test. Correlation was analysed by Spearman's rank correlation coefficients.

Results

Preinfection urinary neopterin concentrations: In both macaque species stable baselines for neopterin were measured with an interindividual variation of 20-50 %. The mean physiological neopterin concentrations in cynomolgus macaques of 259.3 (S.D. ± 132.7 $\mu\text{mol/mol}$ creatinine) were more than two times higher than concentrations found in rhesus monkeys (115.4 ± 38.6 $\mu\text{mol/mol}$ creatinine; $p < 0.01$).

Virus isolation and seroconversion: In (Tab. 1) 1 rhesus and cynomolgus monkeys involved in this study; the respective virus strains used for inoculation; the corresponding virus dilutions injected and the outcome of infection are listed. All rhesus monkeys infected either with SIVagmTYO-1 or with SIVmac at a dilution of 10^{-2} and one of two animals which received SIVmac diluted to 10^{-4} seroconverted within 4 to 6 weeks after infection. The other rhesus monkey receiving SIVmac diluted to 10^{-4} and both animals injected with SIVmac diluted to 10^{-6} remained seronegative. One rhesus monkey inoculated with undiluted HIV-2ben (stock 1) and all cynomolgus macaques inoculated with either undiluted uncloned (stock 1) or clone-derived HIV-2ben, as well as those inoculated with 10^{-4} of the new uncloned HIV-2ben stock (stock 2) seroconverted within 4 weeks after infection. All cynomolgus monkeys inoculated with 10^{-5} or 10^{-6} of the HIV-2ben virus stock 2 did not seroconvert. Virus was reisolated within six weeks after infection (Tab. 1) from all seropositive cynomolgus macaques except for one animal infected with the clone-derived virus. In

Table 1: Response of macaques to infection with SIV or HIV-2

Laboratory investigations												Clinical signs		
Virology						Immunology								
						Neopterin								
Animal		Virus inoculum		Dose	VR ^b	SC ^c	mean % ^d	max % ^e	T4/T8 (↓)	T4 (↓)	T (↓)	L	Ly	D/S ^f
Sp ^a	No.	Isolate												
A: Seropositive macaques														
MM	5025	SIVagmTYO-1 ^g	1	-	6	365	1855	-	-	-	-	-	-	
MM	5026	SIVagmTYO-1	1	4	6	249	2248	-	-	-	-	-	-	
MM	1689	SIVmac251/32H	10 ⁻²	≥ 2	4	305	800	+	+	+	+	+	+	S
MM	1705	SIVmac251/32H	10 ⁻²	≥ 2	4	373	869	+	+	+	+	+	+	D
MM	5024	SIVmac251/32H	10 ⁻²	≥ 4	4	620	2917	+	-	-	+	-	-	
MM	5028	SIVmac251/32H	10 ⁻²	≥ 2	4	700	2162	+	+	+	+	+	-	D
MM	5129	SIVmac251/32H	10 ⁻²	≥ 2	4	450	1571	+	+	+	+	+	-	
MM	5133	SIVmac251/32H	10 ⁻²	≥ 2	4	490	1505	+	+	+	+	+	-	
MM	5019	SIVmac251/32H	10 ⁻⁴	≥ 4	4	712	3739	+	+	+	+	+	-	
MM	5020	HIV-2ben	1	-	4	192	405	-	-	-	-	-	-	
MF	5172	HIV-2ben	1	4-20	4	200	392	-	-	-	-	-	-	
MF	5173	HIV-2ben	1	2-20	4	106	178	-	-	-	-	-	-	
MF	5344	HIV-2ben	10 ⁻⁴	2-32	4	92	121	-	-	-	-	-	-	
MF	5345	HIV-2ben	10 ⁻⁴	≥ 4	4	148	293	-	-	-	-	-	-	
MF	5346	HIV-2ben	10 ⁻⁴	2-24	4	98	194	-	-	-	-	-	-	
MF	5170	HIV-2benMK6 ^h	1	-	4	91	130	-	-	-	-	-	-	
MF	5171	HIV-2benMK6	1	2-4	4	225	418	-	-	-	-	-	-	
B: Seropositive macaques														
MM	5018	SIVmac	10 ⁻⁴	-	-	136	260	-	-	-	-	-	-	
MM	5022	SIVmac	10 ⁻⁶	-	-	110	477	-	-	-	-	-	-	
MM	5023	SIVmac	10 ⁻⁶	-	-	122	176	-	-	-	-	-	-	
MM	5021	HIV-2ben	1	-	-	201	384	-	-	-	-	-	-	
MM	5348	HIV-2ben	10 ⁻⁵	-	-	81	98	-	-	-	-	-	-	
MM	5353	HIV-2ben	10 ⁻⁵	-	-	101	176	-	-	-	-	-	-	
MM	5355	HIV-2ben	10 ⁻⁵	-	-	91	181	-	-	-	-	-	-	
MM	5357	HIV-2ben	10 ⁻⁶	-	-	83	140	-	-	-	-	-	-	
MM	5358	HIV-2ben	10 ⁻⁶	-	-	76	128	-	-	-	-	-	-	

^aSp, species; MM, *Macaca mulatta*; MF, *Macaca fascicularis*^bVR, virus recovery (weeks after infection)^cSC, seroconversion (weeks after infection)^dmean %, mean neopterin concentration within 40 days after inoculation (% of baseline)^emax %, maximum neopterin concentration after inoculation (% of baseline)^fT4/T8, T4/T8 cell ratio; ↓, decrease of; T4, absolute T4 cells; L, lymphadenopathy; T, thrombocytopenia;

↓, decrease of; abs, absolute; Ly, lymphoma; D, death; S, sacrificed

^gisolated from animal 5264 10 weeks after infection (16)^hcell cultured expanded molecular clone of HIV-2ben

contrast to both SIVagm-infected monkeys, from which only one was virus positive at one occasion, virus was reisolated from all SIVmac-infected monkeys at each attempt. Despite seroconversion, virus could neither be recovered from one HIV-2-infected rhesus monkey during the whole observation period. In the majority of the animals cell-associated viraemia preceded seroconversion. Virus could not be recovered

from any inoculated monkey remaining seronegative.

Neopterin as a very early response to the infection: In seropositive animals the mean values of the neopterin concentrations measured within 40 days after infection ranged from 91% to 712% of the baseline. The maximum values ranged from 121% to 3739 % (Tab. 1). In seronegative monkeys mean values ranged from 76% to 201% and maximum increas-

Figure 1: Course of urinary neopterin concentrations (mean $\pm$ S.E.M.) in rhesus and cynomolgus monkeys after inoculation with SIV or HIV-2. Neopterin concentrations obtained after inoculation were related to preinoculation values and expressed as percent of mean baseline values. The diagram shows the mean neopterin concentrations of (A) rhesus monkeys after infection with SIV ($n=9$; animal No. 5025, 5026, 1689, 1705, 5024, 5028, 5129, 5133, 5019 from Table 1) and of (B) cynomolgus monkeys after infection with HIV-2ben ($n=7$; animal No. 5172, 5173, 5344, 5345, 5346, 5170, 5171 from Table 1).

cs from 98% to 477%. No significant differences were found between the preinfection neopterin concentrations of seroconverted and non-seroconverted animals.

The course of mean neopterin concentrations of rhesus monkeys inoculated with SIV is shown in Fig. 1A. Average neopterin concentrations were increased tenfold. In 8 of 9 SIV-seropositive monkeys neopterin concentrations were already increased over baseline levels between days 6 to 10 after infection ($p = 0.013$, Wilcoxon paired rank test). 11 to 15 days after

infection, all SIV positive monkeys showed peak neopterin levels which were dropping thereafter but remained elevated at least three times above the baseline until the end of the 40-days observation period ($p < 0.01$). In contrast, cynomolgus macaques infected with HIV-2 (Fig. 1B) exhibited only mean values two times above preinfection values. In all these monkeys the urinary neopterin concentrations were increased significantly from days 15 to 20 after infection. Concentrations had already declined to baseline levels at the end of the study. In the two rhesus monkeys (# 5020 and # 5021) inoculated with undiluted HIV-2 (stock 1) also a weak increase like in the HIV-2-infected cynomolgus monkeys was monitored exceeding the baseline values only up to four times (Tab. 1).

Three rhesus monkeys inoculated with the higher dilutions of SIVmac remained seronegative. The mean neopterin concentrations of this group after inoculation are shown in Fig. 2A demonstrating a mean increase of two times of the baseline level. One rhesus monkey receiving the 10^{-6} virus dilution (# 5022) showed a significant increase between days 15 to 20 postinoculation, whereas the other two macaques either inoculated with 10^{-4} (# 5018) or 10^{-6} (# 5023) showed only slight increases of their neopterin concentrations (Tab. 1). In contrast, in cynomolgus monkeys remaining virus and antibody negative after HIV-2ben inoculation the urinary neopterin concentrations were not elevated (Fig. 2B). For HIV-2ben-infected monkeys the virus dose correlated significantly with neopterin concentrations measured postinfection (mean in % baseline: $r_s = 0.748$, $p < 0.01$, Spearman rank correlation).

Rise of neopterin precedes clinical disease progression: All monkeys which had seroconverted after inoculation with SIVmac32H developed a persistent lymphadenopathy of their peripheral lymph nodes. Furthermore, in all these animals a decrease of the CD4⁺/CD8⁺ ratio and of the absolute CD4⁺ counts (except # 5024) was noted following infection (Tab. 1). Blastogenic responses of the PBMC from these infected macaques to phytohemagglutinin (PHA) or concanavalin A were also impaired in comparison with preinfection values and negative controls (data not shown). A severe thrombocytopenia occurred as early as 12 weeks after infection in rhesus monkeys of Indian origin (# 1689 and # 1705) and in nearly all the others originating from China approximately 3-6 months later. Two monkeys of the SIVmac32H group have died from AIDS, one 8 months after infection showing multiple lymphomas (# 1705), the other 15 months postinoculation presented with generalized lymphadenopathy. Another monkey (# 1689) developed severe multiple lymphomas and had to be

Figure 2: Course of urinary neopterin concentrations (mean $\pm$ S.E.M.) in rhesus and cynomolgus monkeys after inoculation with SIV or HIV-2. Neopterin concentrations obtained after inoculation were related to preinoculation values and expressed as percent of mean baseline values. The diagram shows the mean neopterin concentrations of (A) rhesus monkeys remaining seronegative after inoculation with SIV ($n=3$, animal No. 5018 - open squares, 5022 - open circles, 5023 - filled triangles - from Table 1) and of (B) cynomolgus monkeys remaining seronegative after inoculation with HIV-2 ($n=5$; animal No. 5348, 5353, 5355, 5357, 5358 from Table 1). Note the scale difference on the Y-axes.

euthanised 11 months after infection. In contrast, the two rhesus macaques receiving the *in vivo* passage of SIVagmTYO-1 and one rhesus monkey and all cynomolgus monkeys infected with clone-derived or uncloned HIV-2ben remained apparently healthy through 14 to 16 months after inoculation. In general, a higher increase of neopterin always preceded clinical changes and alterations of blood parameters indicating

immunodeficiency. In the monkeys infected with SIVagmTYO-1 and remaining asymptomatic neopterin appeared to reach the baseline level faster, whereas in the SIVmac-infected macaques showing signs of immunodeficiency neopterin remained on an elevated level during the observation period (data not shown).

Discussion

In both macaque species stable baseline values of urinary neopterin concentrations were noted for each individual before infection. Significantly lower physiological neopterin concentrations were observed in rhesus monkeys compared to cynomolgus macaques. Seroconversion after experimental infection of rhesus and cynomolgus macaques could be demonstrated within 6 weeks after infection. For both, the SIVmac251/32H and the HIV-2ben virus stock, a very similar *in vivo* titer could be found, confirming for SIVmac251/32H the endpoint already described (20,23).

Due to infection of rhesus and cynomolgus macaques with SIV and HIV-2, respectively, neopterin concentrations increased significantly and always preceded seroconversion. The increase during the acute phase of infection corresponds well with cytokine mRNA expression in mononuclear cells during acute SIVmac251 infection of macaques (24).

Interestingly, in all SIV-infected rhesus macaques the mean neopterin concentrations increased 12-times over baseline level within 11-15 days after infection. In HIV-2 seropositive cynomolgus macaques the increase was somewhat delayed and only threefold. These data raise the question whether this distinct response is due to the macaque species inoculated or to biological properties of the viruses. Two further observations support the latter explanation. Firstly, in one cynomolgus monkey infected with 1 mL of a 10^{-2} of SIVmac251/32H the neopterin concentrations were still elevated four times above the baseline level 30 to 40 days after infection (data not shown). In contrast, at the same time point neopterin concentrations in all HIV-2ben-infected cynomolgus monkeys were no longer significantly elevated. Secondly, the neopterin concentrations in the one HIV-2ben-infected rhesus monkey (Tab. 1) resemble those reported earlier for two rhesus monkeys infected with SIVmac251 which in our hands was only low pathogenic (15). Thus, the apathogenic HIV-2ben as well as the low pathogenic SIVmac isolate only induced a weak immune activation. This is indicated by a comparably low and short-term increase of neopterin in both monkey species. In contrast, the pathogenic SIVmac251/32H with a high replicative capacity *in vivo* as indicated by persistent

cell-associated viraemia (Tab. 1) led to a higher and faster increase of neopterin remaining elevated even 40 days post-infection. These data reflect the situation observed in HIV-1-infected humans (25). Data on neopterin increase in CSF, but not in serum of D-type retrovirus infected rhesus monkeys (17) further support our assertion that the differences in the elevation of the neopterin levels are virus related.

However, in addition to the virus isolate-dependent increase of neopterin concentrations there also seem to be species-specific reactions. In rhesus monkeys remaining virus and antibody negative after SIVmac inoculation neopterin concentrations increased slightly. Apart from a different immune status of each individual not yet definable, another reason could be the stimulus of a subinfectious viral dose. At present it is not known whether these animals became infected by SIV without measurable seroconversion and virus recovery during the whole follow-up. To clarify the viral status further studies are needed using more sensitive methods such as the polymerase chain reaction (26). On the other hand, according to the *in vitro* titer of the SIVmac251/32H pool in C81-66 cells of 104 TCID₅₀/mL (data not shown) used for inoculation it is highly probable that infectious virus had been diluted-out. This obviously unspecific effect was not seen in HIV-2 inoculated seronegative cynomolgus macaques. So far, our data do not allow to conclude whether this phenomenon is due to the immune status of the individual animal or to inherent biological properties of the inoculated virus e.g. infectivity, concentration or replicative efficiency. However, at least a simple antigenic stimulation provided by the fetal calf serum present in the inoculate can be excluded. In six naive rhesus macaques receiving 1 mL of cell-culture supernatant from non-infected C81-66 cells containing 10 % fetal calf serum no neopterin elevations were measured over 30 days following inoculation (data not shown) thereby supporting our earlier findings (15).

Nevertheless, in all SIVmac251/32H-infected monkeys the high increases in neopterin concentration very early after infection were closely correlated with typical signs of immunodeficiency occurring in later stages of infection. Thus, our findings agree with those described for the early phase of HIV-1-infection in man (10,11), and also an association between a high initial neopterin increase followed by an accelerated loss of CD4⁺ T-cells has been described in HIV-infected humans (27). Also neopterin determined in various body fluids from HIV-1 seropositive patients has been shown to be a powerful independent predictor of disease progression (4,28,29).

Our findings about the early phase after infection of macaques with SIV or HIV-2 are not only strikingly

similar, but moreover exceed data about HIV-1-infection in man. Whereas we could demonstrate a causative relationship between the time point of infection and the occurrence of the initial neopterin peak 10-14 days after infection, in HIV-1-infected humans the time point of infection very often can only be vaguely estimated retrospectively (9,10). Therefore, elevated neopterin concentrations cannot necessarily be interpreted as a consequence of infection. In these cases one could only speculate about the nature of this correlation, since preinfection values could not be obtained and other acute viral and non-viral infections are also accompanied by high neopterin levels (5).

In our SIV- or HIV-2-infected monkeys urinary neopterin is a very useful adjunct to virus isolation and seroconversion to demonstrate infection because it is sensitive, rises very early and can easily be measured and quantified. Moreover, it predicts disease progression. In addition, this marker coincides with metabolic disorders of the immune system described very recently for SIV-infected macaques (30). Therefore, we suggest to include neopterin as marker in study protocols to monitor therapeutic effects of potential anti-retroviral drugs in SIV- or HIV-2-infected macaques as described in humans (29,31). This marker should also indicate a therapy failure due to the appearance of therapy-resistant mutants. This would enable clinicians to quickly change the therapeutic strategy. Moreover, neopterin is a sensitive and early indicator for infection in experimental vaccine studies (32). Therefore, we recommend to include this parameter in therapeutic, prophylactic and pathogenesis studies. In recent studies it turned out that the determination of neopterin still has its place even when quantitative HIV RNA measurements with polymerase chain reaction are performed (8,33). Moreover, urine sampling is easier and safer than venipuncture regarding HIV transmissibility (34), and neopterin concentrations remain stable for several days at ambient temperature without additional precautions.

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