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Neopterin modulates toxicity mediated by reactive oxygen and chloride species

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Neopterin, a pyrazino-pyrimidine derivative, is synthesized in excess by human monocytes/macrophages upon stimulation with interferon-γ, a cytokine derived from activated T-cells. Neopterin is furthermore produced constitutively. A relatively constant ratio between neopterin and its reduced form, 7,8-dihydroneopterin, has been described in human serum. In the study presented here we tested the ability of neopterin and its reduced form to modulate the effects of cytotoxic substances like hydrogen peroxide or hypochlorous acid and *N*-chloramine derivatives. We show that 7,8-dihydroneopterin potently reduces biological and chemical effects of these substances independently from the pH value. In contrast, at slightly alkaline pH (pH 7.5) neopterin enhances hydrogen peroxide and chloramine-T activity. This is demonstrated by increase of signal intensity in a luminol assay and also by enhancement of toxicity towards bacteria. Thus, the macrophage derived substance neopterin is able both to enhance and to reduce cytotoxicity in dependence of pH value and its oxidation state, and it may have a pivotal role in modulation of macrophage mediated effector mechanism.

Neopterin; Antimicrobial toxicity; Macrophage; Hydrogen peroxide; Chloramine

1. INTRODUCTION

The macrophage is a central effector cell of the cellular immune response. When stimulated by cytokines derived from activated T-cells such as, e.g. interferon-γ, macrophages produce, besides a lot of other secretory products, reactive metabolites of oxygen such as hydrogen peroxide, superoxide and hydroxyl radicals [1]. Hydrogen peroxide is a central molecule in mediating macrophage and granulocyte mediated extracellular cytolysis [2]. Upon stimulation, monocytes further release myeloperoxidase which reacts with hydrogen peroxide and chloride to form hypochlorous acid and chloramines. These components have turned out to be highly toxic to ingested microorganisms [3,4]. Upon stimulation by interferon-γ, macrophages further produce and release large amounts of neopterin [5], and concentrations up to 1 μmol/l have been measured locally in humans [6]. Neopterin derives from 7,8-dihydroneopterin triphosphate, the first intermediate in the biosynthesis of tetrahydrobiopterin from GTP. Since human macrophages contain a particularly low activity of 6-pyruvoyl tetrahydropterin synthase, the second enzyme of the pathway, only a minor of the accumulated 7,8-dihydroneopterin triphosphate is further metabolized to tetrahydrobiopterin, whereas the major part is cleaved by phosphatases to 7,8-dihydroneopterin [7]. This is then partially oxidized to neopterin and a nearly constant ratio neopterin to dihydroneopterin of about 1:2 has been observed in serum of healthy individuals, corresponding to median concentrations of 4.7 nmol/l neopterin and 8.9 nmol/l 7,8-dihydroneopterin [8]. Although neopterin has turned out as a clinically useful marker for monitoring patients with activated cellular immunity [9], e.g. elevated neopterin levels were observed in subjects suffering from viral infections like hepatitis or HIV-infection, malignancies, autoimmune disorders or during allograft rejection [10–14], no biological role could be established so far for this substance.

Neopterin is furthermore produced constitutively, and most recently significantly higher concentrations of 7,8-dihydroneopterin were observed in arterial as compared to venous blood [15]. This finding suggests that these alterations of neopterin levels may be due to processes occurring in the peripheral capillary system. Since neither neopterin nor 7,8-dihydroneopterin did exert an effect on the binding of oxygen to hemoglobin, the uptake of carbon dioxide by erythrocytes, or the activity of enzymes such as peroxidase (Weiss, G. et al., unpublished observations), we tested whether neopterin and 7,8-dihydroneopterin may influence effects of hydrogen peroxide, hypochlorous acid and chloramines.

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2. MATERIALS AND METHODS

2.1. *Luminol-dependent measurement of chemiluminescence*

Neopterin, 7,8-dihydroneopterin and 5,6,7,8-tetrahydroneopterin (Schircks Lab., Jona, Switzerland) were dissolved in phosphate-buffered saline which was previously adjusted to pH between 5.5 and 7.5. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione obtained from Sigma, Munich, Germany) was dissolved in DMSO at a final concentration of 10 mM and a 40 μM working solution was freshly prepared by dilution with phosphate-buffered saline, pH 7.5. 150 μl of luminol solution and 750 μl of phosphate-buffered saline with/without variable amounts of pteridines were incubated in microcuvettes at 37°C in a luminometer (LKB 1251, Pharmacia, Piscataway, NJ). 200 μl of 1% hydrogen peroxide (0.29 M) or 0.05% chloramine-T (1.77 mM) solution were injected and light output was measured for 30 s at 37°C. Maximum light output of samples was compared to that of control (phosphate buffered saline, luminol, chloramine-T or hydrogen peroxide). Plots show mean values (mean ± S.E.M.) for four independent experiments performed in duplicate.

2.2. *Quantification of antimicrobial toxicity*

Bacteria of the strains *Escherichia coli* and *Staphylococcus aureus* were grown in Nutrient Broth No. 2 medium (Oxoid Ltd., Hants, UK) for 24 h. 200 μl of this suspension of exponentially growing bacteria were diluted in 50 ml of fresh culture medium. One ml was transferred to sterile cuvettes containing 1 ml of phosphate-buffered saline (pH 7.5) with/without neopterin or 7,8-dihydroneopterin (final concentration 200 μM). Then 1 ml 0.25% (8.9 mM) chloramine-T was added and cytotoxicity towards bacteria was measured by poured plate technique. This was carried out by transferring 500 μl of bacteria/phosphate-buffered saline with/without neopterin or 7,8-dihydroneopterin/chloramine-T suspension to a sterilized plate and further addition of 10 ml of molten nutrient agar. All plates were incubated for 24 h at 37°C.

3. RESULTS AND DISCUSSION

As pointed out in Fig. 1, 7,8-dihydroneopterin as well as 5,6,7,8-tetrahydroneopterin drastically reduce maximum chloramine-T, a disinfectant dissociating in aqueous solution into hypochloric acid and various *N*-chloramines [16], and hydrogen peroxide-induced chemoluminescence in a luminol assay. The reduction occurred in a dose–response relationship and was almost unchanged within the pH range tested (from pH 5.5 to 7.5). 7,8-Dihydroneopterin and 5,6,7,8-tetrahydroneopterin are almost equal in their scavenging activity.

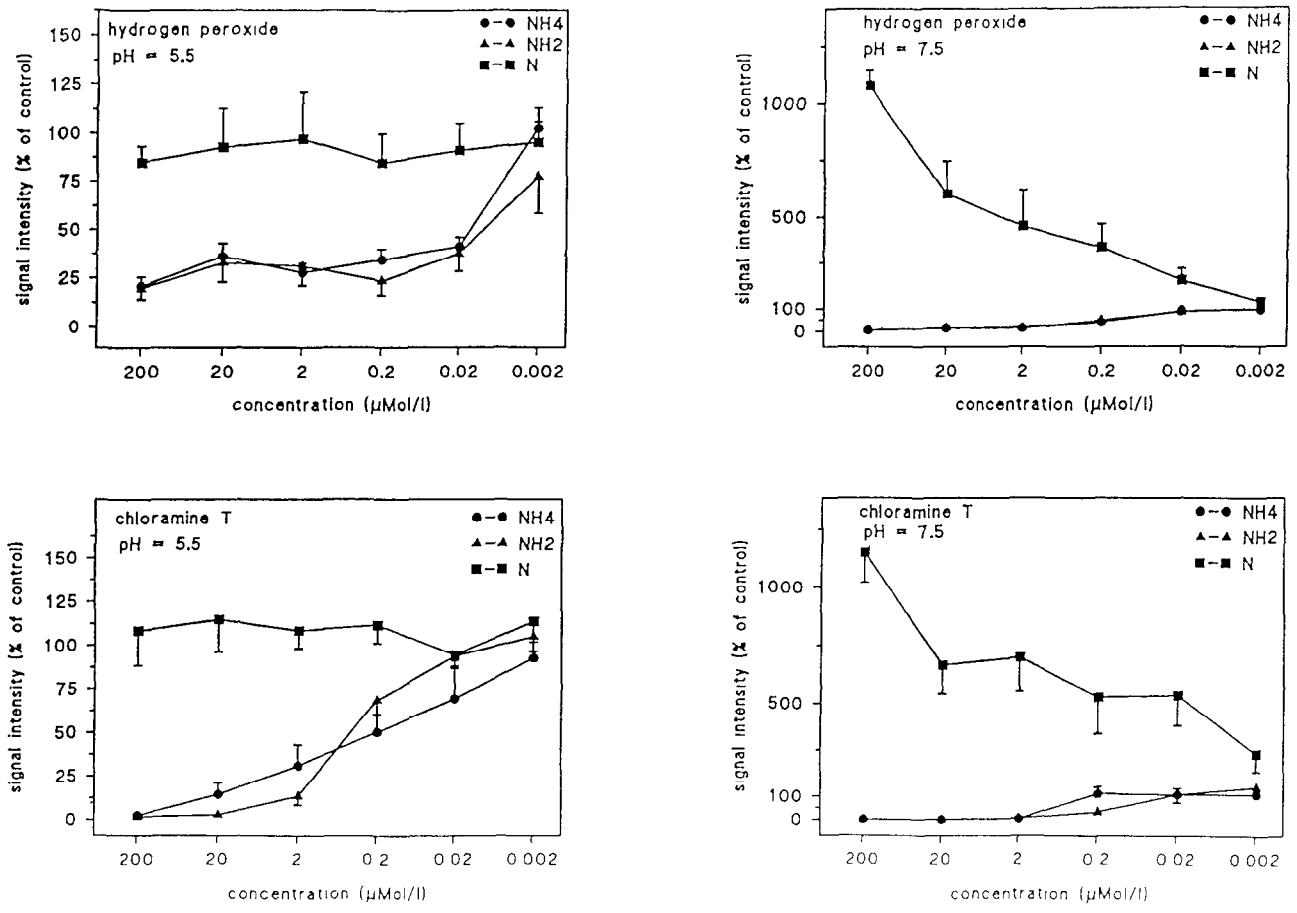

Fig. 1. Effects of pteridines on hydrogen peroxide- and chloramine-T-induced chemoluminescence in dependence on pH-value. The upper panel shows effects of increasing concentrations of neopterin (N), 7,8-dihydroneopterin (NH2), and 5,6,7,8-tetrahydroneopterin (NH4) on hydrogen peroxide-induced luminol-dependent chemoluminescence. Light output after addition of hydrogen peroxide (final concentration 53 mM) to luminol and phosphate buffered saline solution was used as control. The peak value of light output of the control was set equal to 100% and compared with peak values of the samples investigated. Since in all experiments the results obtained at pH 5.5 and pH 6.5 were not significantly different, only results obtained at pH 5.5 are shown. The lower panel outlines the results for the corresponding experiments performed with chloramine-T (final concentration 320 μM).

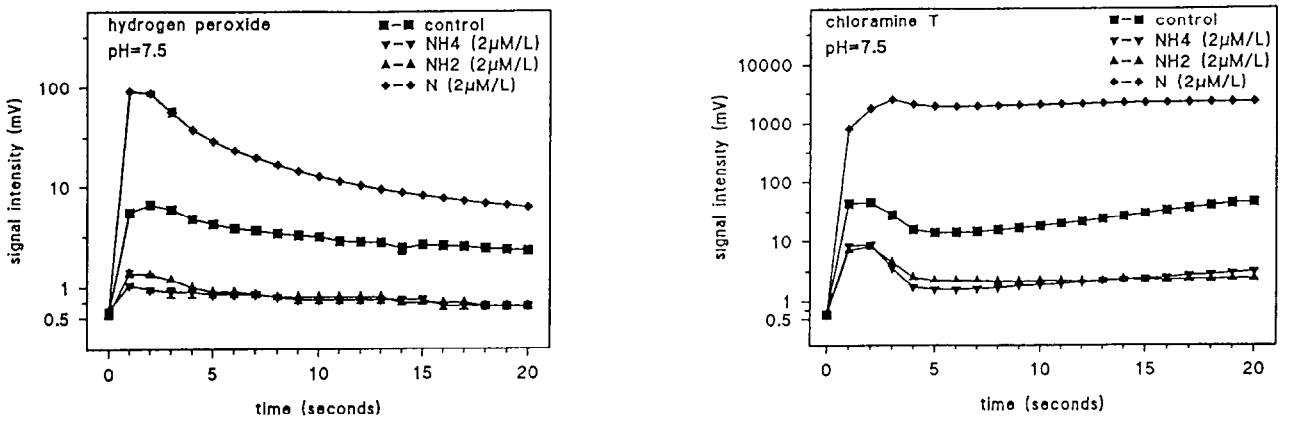

Fig. 2. Time course of luminol-dependent chemoluminescence in the presence of reactive molecules and pteridines at pH 7.5. Oxidant concentrations were the same as described in the legend to Fig. 1.

In contrast, neopterin did not significantly alter chloramine-T or hydrogen peroxide induced signal intensity at slightly acidic pH (5.5 and 6.5), but had an exorbitantly enhancing effect on chemoluminescence at pH 7.5, also following a dose–response relationship (Fig. 1).

Fig. 2 outlines the time course of luminol-dependent chemoluminescence. In the presence of a medium neopterin concentration (2 μM) a sharp increase of hydrogen peroxide (left) as well as chloramine-T (right)-induced signal intensity occurs (Fig. 2). This multiplication of light output is not delayed in time as compared with the control (hydrogen peroxide or chloramine-T alone), is observed immediately after addition of chloramine-T/hydrogen peroxide and therefore does not seem to be due to, e.g. time-dependent accumulation of reactive molecules in the presence of neopterin. This suggests that neopterin may enhance formation of reactive molecules originating from chloramine-T or hydrogen peroxide or, that neopterin itself may be converted into a reactive intermediate upon reaction with hydrogen peroxide and chloramine-T. On the other hand, 7,8-dihydroneopterin and 5,6,7,8-tetrahydroneopterin reduce signal intensity as compared with the control (Fig. 2). This is likely due to effective scavenging of reactive molecules derived from hydrogen peroxide or chloramine-T. It can be further seen that neopterin, 7,8-dihydroneopterin and 5,6,7,8-tetrahydroneopterin per se do not cause any change of luminol-dependent chemoluminescence since basic levels (at time 0, i.e. before injection of chloramine-T or hydrogen peroxide) are not different from the control, i.e. luminol and phosphate-buffered saline, pH 7.5.

We then tested if the pteridine-mediated modulation of chemoluminescence may influence toxicity of hydrogen peroxide or chloramine-T against microorganisms. Fig. 3 depicts the results of a typical pouring plate experiment performed with *E. coli* bacteria in the presence of chloramine-T and/or neopterin or 7,8-dihydroneop-

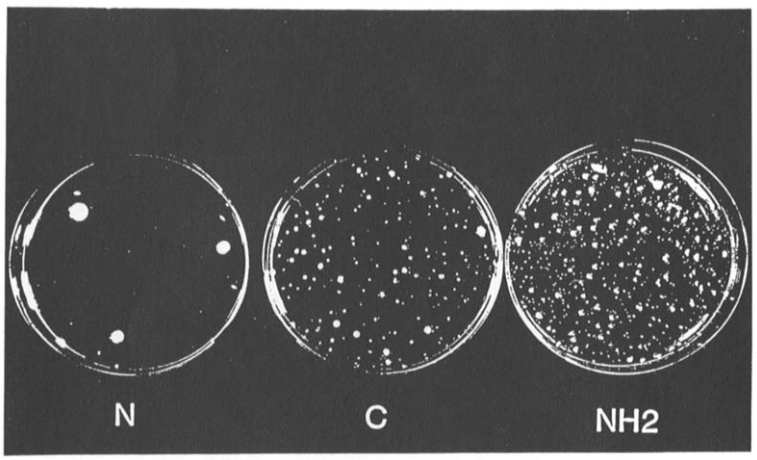

Fig. 3. Alteration of chloramine-T-induced toxicity towards microorganisms by neopterin and 7,8-dihydroneopterin as tested by survival of *E. coli*. After addition of pteridines and chloramine-T bacteria were incubated at 37°C for 30 min. Pouring plate technique was used for quantification of bacteria survival. C, control (bacteria, chloramine-T, phosphate-buffered saline, pH 7.5); N, control plus neopterin; NH2, control plus 7,8-dihydroneopterin. For details see section 2.

terin. From these data it is evident that exposure of bacteria to neopterin and chloramine-T drastically reduces the number of colonies as compared to treatment with chloramine-T alone. On the other hand, concomitant administration of 7,8-dihydroneopterin and chloramine-T to *E. coli* enhanced survival of bacteria as compared to chloramine-T control. This suggests that oxidized neopterin is a potent enhancer and multiplier of cytotoxic effects of hydrogen peroxide and chloramine-T, while 7,8-dihydroneopterin is a potent scavenger. The results were almost the same when using *Staph. aureus* for testing of toxicity, demonstrating that effects of neopterin and 7,8-dihydroneopterin on chloramine-T mediated cytotoxicity are not specific for *E. coli* (details not shown). In contrast, bacterial growth was not altered after sole addition of 7,8-dihydroneopterin or neopterin (200 μM final concentration) to *E. coli* as compared with a control (bacteria supplemented with phosphate-buffered saline; details not shown). While the observation of scavenger activity for reduced pteridines is in good accordance with previously published reports [17,18], the potentiating effect of oxidized neopterin and its pH dependence have not been described hitherto. Since neopterin and 7,8-dihydroneopterin are concomitantly synthesized with hydrogen peroxide by interferon-γ stimulated macrophages [19], our observations suggest that neopterin/dihydroneopterin modulate macrophage induced cytotoxicity either way. The ratio of neopterin to 7,8-dihydroneopterin may determine whether hydrogen peroxide or hypochlorous acid induced effects are potentiated or reduced. From our data, it is tempting to speculate that macrophages increase extracellular cytotoxicity by enhanced generation, release and oxidation of 7,8-dihydroneopterin to form neopterin while they protect themselves from being damaged by intracellular accumulation of 7,8-dihydroneopterin. In a variety of clinical conditions activated macrophages and their secretory products contribute to tissue damage. This is most evident in patients with autoimmune disorders, allograft rejection, but also during infection. In all these clinical conditions increased neopterin levels have been found to correlate with the severity of the disease. Our findings suggest a potential role of neopterin in modulating macrophage-induced extracellular toxicity in dependence of pH and the relative ratio of neopterin to 7,8-dihydroneopterin. Our data may explain why activated macrophages produce large amounts of neopterin, namely, to further enhance their cytotoxic potential.

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