Comparative Effects of Heme and Metalloporphyrins on Interferon-γ-Mediated Pathways in Monocytic Cells (THP-1) (43561)

GUNTER WEISS,* JOHN D. LUTTON,† DIETMAR FUCHS,* GABRIELE WERNER-FELMAYER,* GUNTER BOCK,‡ NADER G. ABRAHAM,†,§,1 ATTALLAH KAPPAS,§ RICHARD D. LEVERE,†,§ AND HELMUT WACHTER*

Institute for Medical Chemistry and Biochemistry, University of Innsbruck, A-6020 Innsbruck, Austria; Department of Medicine,† New York Medical College, Valhalla, New York 10595; Institute for General and Experimental Pathology,‡ University of Innsbruck, A-6020 Innsbruck, Austria; and The Rockefeller University,§ New York, New York 10021*

Abstract. **Previous results have demonstrated links between cell-mediated immunity, interferon (IFN)-γ and neopterin production with heme, porphyrins, and iron metabolism. In this study, we compared the effects of heme, several metalloporphyrins, protoporphyrin IX, and iron on the signal or IFN-γ-mediated pathways, such as the expression of major histocompatability complex class II antigens, neopterin formation, and the degradation of tryptophan. Using the human monocytic cell line, THP-1, we found that heme, Zn-mesoporphyrin, Zn-deuteroporphyrin, Co-protoporphyrin, and iron reduced the efficiency of the IFN-γ signal. In addition, Zn-mesoporphyrin almost fully inhibited IFN-γ-induced degradation of tryptophan by the heme protein, indoleamine 2,3-dioxygenase. In contrast, tin-protoporphyrin enhanced the IFN-γ effects as seen by increased neopterin production, enhanced tryptophan degradation, and elevated HLA-DR antigen expression on cells. These effects are considered to be due to the action of heme, metalloporphyrins, iron, or heme byproducts on the IFN-γ signal, rather than to direct effects on IFN-γ-induced enzymatic pathways. Heme and metalloporphyrins were previously shown to affect heme oxygenase activity, T cell growth, and lipid peroxidation and to modulate interleukin 2 activity. These pathways are also known to be influenced by IFN-γ, and our data suggest that heme and metalloporphyrins may directly modulate the efficiency of the IFN-γ signal.** [P.S.E.B.M. 1993, Vol 202]

Previously, we demonstrated a close correlation between increased urinary concentrations of total porphyrins, porphobilinogen, and neopterin in human immunodeficiency virus (HIV)-1 infection (1). Furthermore, it has been reported that in some instances the acquired immune deficiency syndrome is associated with a deranged porphyrin metabolism (2). *In vivo* determination of neopterin has proved to be useful clinically as a diagnostic and prognostic tool for monitoring patients with diseases involving the cellular immune system, e.g., patients with various infections or neoplasia or recipients of allografts (3–6).

Under certain conditions, activation of the immune system results in neopterin production by monocytes or macrophages, and the production is stimulated by exposure to interferon (IFN)-γ (7). The biosynthesis of neopterin is dependent upon the conversion of guanosine triphosphate to several pteridine derivatives such as neopterin, 7,8-dihydroneopterin, and 5,6,7,8-tetrahydrobiopterin (8). The latter compound is an essential cofactor for neurotransmitter synthesis (9) and nitric oxide-synthase (10, 11).

In parallel with neopterin synthesis, IFN-γ stimulates the degradation of tryptophan and other indoleamine derivatives to kynurenine, anthranilic acid, and 3-hydroxyanthranilic acid, and this is due to the direct induction of indoleamine 2,3-dioxygenase (12, 13). This mechanism is also of interest *in vivo*, since it was shown that chronic immune activation is associated with reduced levels of tryptophan and increased kynurenine concentrations (14).

Heme and several metalloporphyrins, i.e., substi-

[1] To whom requests for reprints should be addressed at Department of Medicine, New York Medical College, Valhalla, NY 10595.

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tution of other metals to the porphyrin nucleus, have attracted great clinical interest in a variety of disorders (15). Since there are correlations between porphyrins and infectious states, heme and various metalloporphyrins may be pertinent to the effects of IFN-γ and neopterin. Heme is a natural regulator of cellular functions, and disturbances in heme metabolism may account for numerous disorders (16, 17). Heme has been shown to suppress HIV replication and to enhance the antiviral effect of azidothymidine (18). Recently, tin-protoporphyrin (SnPP) has been found to be a potent competitive inhibitor of heme oxygenase (19), the key enzyme for the degradation of heme to bilirubin (20). This has since proved to be useful in the treatment of hyperbilirubinemia (21). Iron released by the degradation of heme may then be used for other cellular processes. It was further shown that metalloporphyrins induce mitogenicity in peripheral T cells and also stimulate the production of tumor necrosis factor-α and IFN-γ by peripheral blood mononuclear cells in synergism with interleukin 2 (22). Similar effects were also described for ferric iron in the presence of interleukin 2 (23). In contrast, several porphyrins exhibit antiproliferative effects toward mouse spleen lymphocytes (24). In a previous study, we found that ferric iron, as well as transferrin-bound iron, drastically inhibits the IFN-γ-mediated effects in the human monocytic cell line, THP-1 (25). Therefore, we were interested in determining whether heme and other metalloporphyrins may affect or interfere with the effectiveness of IFN-γ or its signal.

Materials and Methods

Materials. Human recombinant IFN-γ with a sp act of 2×10^7 units/mg of protein was a generous gift of Bioferon (Laupheim, Germany). Hemin was obtained from Sigma (Munich, Germany). Metalloporphyrins, i.e., Zn-mesoporphyrin (ZnMP), Zn-deuteroporphyrin (ZnDP), SnPP, Co-protoporphyrin (CoPP), and protoporphyrin (PP) were from Porphyrin Products, Inc. (Logan, UT). Two milligrams of ZnMP and PP were dissolved in 2 ml of acidic solution, i.e., 0.1 *M* and 2.7 *M* hydrochloric acid, respectively. Five milliliters of 2% Tris/HCl were added, and the pH was adjusted to 7.8 by addition of sodium hydroxide. Three and a half milliliters of this solution were mixed with 6.5 ml of RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS). All other porphyrins were dissolved in 0.1 *M* sodium hydroxide and further prepared as described above. The pH was adjusted to 7.8 by addition of hydrochloric acid.

Cell Culture. THP-1, a human monocytic leukemia cell line with characteristics of monocytes/macrophages (26), was obtained from the American Type Culture Corporation (Rockville, MD) and cultured as described (27) using RPMI 1640 supplemented with 10% heat-inactivated FCS (Biochrom, Berlin, Germany), 2 m*M* L-glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. For experiments, cells were seeded at a density of 10^6/ml medium in 24-well plates. The culture media were supplemented with iron nitrate or various porphyrins in solution up to a final concentration of 25 μM, and cells were treated with 300 units/ml of human recombinant IFN-γ for 48 hr. Cells were collected and quantified by means of a Coulter counter, supernatants were harvested by centrifugation, and the pellet was washed twice with 0.9% sodium chloride. Pellets were resuspended in 600 μl of distilled water and frozen at −20°C for 1 hr. After rapid thawing, cell debris was centrifuged at 10,000*g*.

Determination of Neopterin, Tryptophan, and Its Metabolites. Estimation of neopterin in supernatants and in cell extracts was performed after iodine oxidation as described previously (25). Briefly, 100 μl of samples were mixed with 10 μl of 0.1 *M* KI/I_2 and incubated for 30 min in the dark, and excess iodine was destroyed by addition of 10 μl of 0.1 *M* ascorbic acid. Pteridine determination was then carried out by solid-phase extraction over AASP-SCX cartridges (Analytichem, Harbor City, CA) and online elution to a reverse-phase high-performance liquid chromatography column (Lichrosorb Rp-18; Merck, Darmstadt, Germany) as described (28). The detection limit for neopterin was 1 n*M*.

Tryptophan and its metabolites were determined in supernatants by high-performance liquid chromatography with fluorescence and ultraviolet detection as reported (29). Detection limits were 0.5 μM for kynurenine, 5 n*M* for 3-hydroxyanthranilic acid and anthranilic acid, and 10 n*M* for tryptophan. Indoleamine 2,3-dioxygenase activity was calculated as the amount of tryptophan metabolites detected in supernatants in nmol/10^6 cells after 48 hr.

Determination of Major Histocompatibility Complex Class II Antigen Expression. Expression of HLA-DR, a representative for major histocompatibility complex (MHC) class II antigens, was estimated by use of the IgG2a mouse monoclonal antibody from the clone L243 (30) obtained from Becton Dickinson (Mountain View, CA). For isotope control, a mouse IgG2a monoclonal antibody anti-CD-10 (Becton Dickinson) was used. L243 and anti-CD-10 were applied as purified immunoglobulin. Cells were seeded at a density of 10^6/ml in 24-well plates, supplemented with iron or porphyrins (25 μM final concentration), and stimulated with 300 units/ml of rhIFN-γ for 48 hr. Cells were collected and washed twice and aliquots of 10^6 cells were resuspended in 50 μl of Dulbecco's modified Eagles medium (DMEM)/2% FCS/0.1% sodium azide and incubated with the appropriate antibody for 30 min on ice. For reagent control, the first incubation

step was carried out with DMEM/2% FCS/0.1% sodium azide. Incubation was terminated by addition of 1 ml of cold DMEM/2% FCS/0.1% sodium azide, and after washing twice, cells were counterstained with fluorescein isothiocyanate-conjugated anti-mouse-IgG serum and diluted 1/100 in DMEM/2% FCS/0.1% sodium azide. For analysis, a fluorescence-activated cell sorter (FACScan; Becton Dickinson) was used. Fluorescence intensities of 5×10^3 cells were measured for each determination. Data are expressed as mean fluorescence channel in a log scale.

Protein Determination. Protein concentrations of cell extracts were estimated according to Bradford (31), modified for 96-flat bottom microtiter plates using an Anthos 2001 microplate reader (Anthos Labtec., Salzburg, Austria).

Statistical Evaluation. Calculation of statistical significance was carried out by Student's *t* test; *P*-values denote significance between the control, i.e., IFN-γ-stimulated cells supplemented with medium, and cells supplemented with porphyrins or iron and treated with this cytokine.

Results

The Effects of Heme and Metalloporphyrins on Neopterin Formation. Fe, Zn, Co, SnPP, PP, and ferric iron were investigated for their effects on IFN-γ-induced neopterin formation. As can be seen in Figure 1, the results obtained from supernatants correlate closely to intracellularly measured neopterin levels of THP-1 cells. Results demonstrate that heme, ZnMP, ZnDP, and CoPP reduce IFN-γ-induced neopterin formation by THP-1 cells. As noted previously (25), the most prominent effect was obtained by ferric iron resulting in a mean reduction of neopterin formation to 36% and 43% of control values for supernatants and cell extracts, respectively. In contrast, PP and, to a greater extent, SnPP enhanced hrIFN-γ-induced pteridine synthesis. None of the substances investigated influenced neopterin formation by THP-1 cells in the absence of immunologic stimuli. Basic neopterin levels were 3.3 ± 0.6 nmol/10^6 cells for supernatants and 13.1 ± 2.7 nmol/mg protein for cell extracts (mean ± SD, $n = 5$).

Interference of Metalloporphyrins with Indoleamine 2,3-Dioxygenase Activity. The effects of iron, heme, and other metalloporphyrins on tryptophan degradation are represented in Figure 2. It is known that tryptophan degradation is due to the induction of indoleamine 2,3-dioxygenase by IFN-γ and that kynurenine is the main metabolite formed (6, 12). In our controls, i.e., cells supplemented with medium and stimulated with IFN-γ for 48 hr, about 60% of the tryptophan in supernatants, 8.0 ± 0.5 mole/10^6 cells (mean ± SE), was degraded. Addition of heme, ZnDP, and PP to cells before cytokine stimulation resulted in a moderate inhibition of indoleamine 2,3-dioxygenase

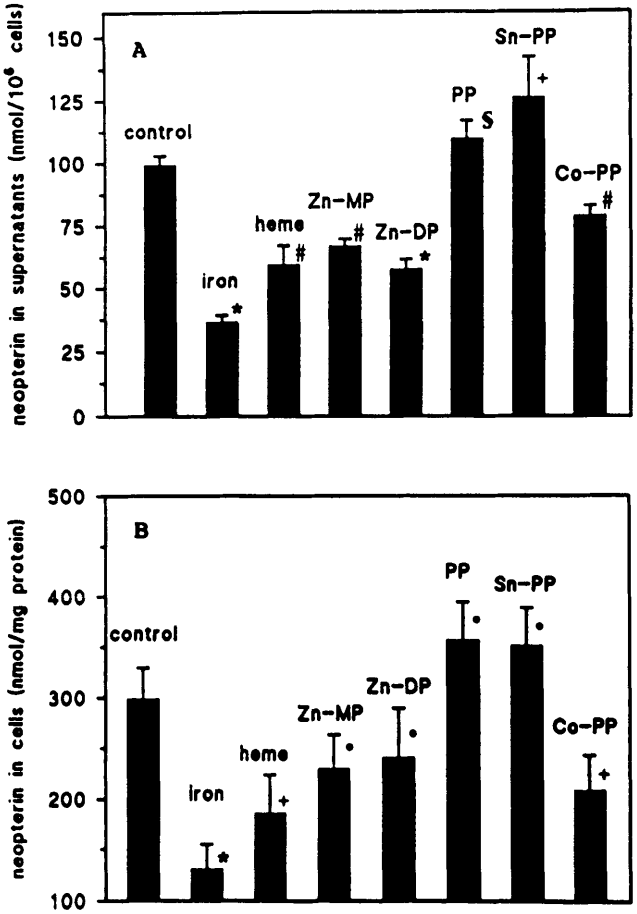

Figure 1. Interference of metalloporphyrins with IFN-γ-induced formation of neopterin. THP-1 cells in RPMI 1640 were supplemented with 25 μM (final concentration) of ferric iron, heme, PP, ZnMP, ZnDP, SnPP, or CoPP and stimulated with hrIFN-γ (300 units/ml) for 48 hr. Cells stimulated with IFN-γ and incubated without supplements were used as control. Neopterin concentrations in (A) supernatants and (B) cell extracts were quantified by means of reverse phase high-performance liquid chromatography as described (25) and referred to cell number and cellular protein content, respectively; $^*P < 0.001$; $\#P < 0.01$; $+P < 0.02$; $^\circ P < 0.05$; $\S P > 0.05$, not significant (with respect to control).

activity, as seen by reduced amounts of tryptophan degraded and kynurenine formed (Fig. 2). A more dramatic inhibitory effect on tryptophan degradation was observed in the presence of ferric iron and CoPP. Furthermore, ZnMP almost fully inhibited indoleamine 2,3-dioxygenase. In contrast, SnPP enhanced indoleamine 2,3-dioxygenase activity as estimated by increased amounts of tryptophan degraded and kynurenine formed.

Influence of Heme and Metalloporphyrins on IFN-γ-Induced MHC Class II Antigen Expression. The effects of these agents on HLA-DR expression, a representative of MHC class II antigens, are presented in Table I. No marked differences in basic HLA-DR expression in the absence of cytokine stimuli were observed between the different groups. However, when cells were stimulated with hrIFN-γ for 48 hr, a dramatic

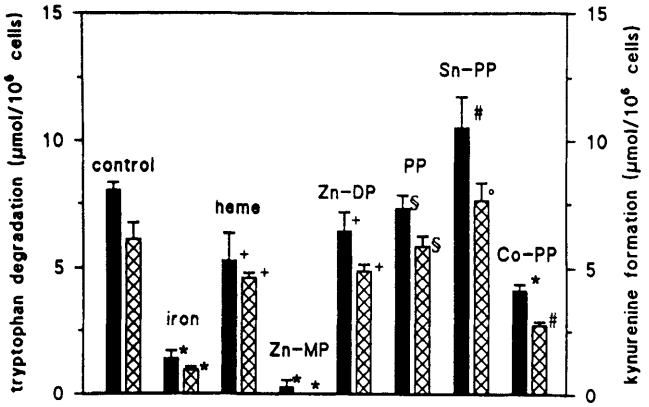

Figure 2. Indoleamine 2,3-dioxygenase activity in the presence of IFN-γ and metalloporphyrins. Cells in RPMI 1640 were supplemented with porphyrins or iron, as described in the legend to Figure 1, and incubated in the presence and absence of 300 units/ml of hrIFN-γ for 48 hr. Control is IFN-γ-treated cells without supplement. Tryptophan degradation was calculated as the difference of tryptophan content in supernatants of unstimulated and IFN-γ-treated cells. The solid bar signifies tryptophan degradation and the crossed bar, kynurenine formation. $^{*}P < 0.001$; $\#P < 0.01$; $+P < 0.02$; $^{\circ}P < 0.05$; $\S P > 0.05$, not significant (with respect to control).

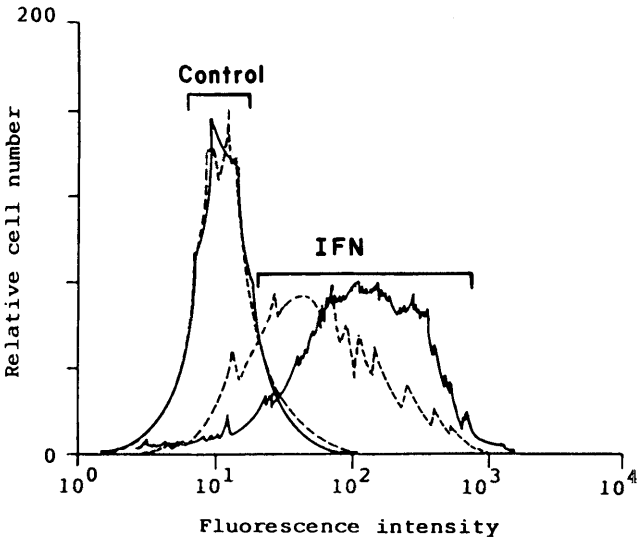

Figure 3. Effect of heme on HLA-DR expression in unstimulated controls (left) (10^0-10^2 range) and IFN-γ-stimulated THP-1 cells (right) analyzed by flow cytometry. Cells were cultured as described in the Materials and Methods section, supplemented with heme—or medium (—) and incubated for 48 hr in the presence and absence of hrIFNγ (300 units/ml).

Table I. Effects of Porphyrins on IFN-γ-Induced Expression of HLA-DR Antigens on THP-1 Cells[a]

Supplement	Log fluorescence intensity		Changes in fluorescence intensity
	Untreated	+IFN-γ	
Medium	13.7 ± 2.9	290.9 ± 20.1	278.9 ± 17.2
Iron	12.6 ± 3.7§	95.7 ± 11.2*	82.6 ± 10.3*
Heme	15.4 ± 3.1§	132.3 ± 8.4*	115.0 ± 8.1*
ZnMP	6.1 ± 3.0‡	237.5 ± 17.2‡	232.0 ± 18.7‡
ZnDP	16.2 ± 2.7§	219.6 ± 26.9†	204.9 ± 28.2†
PP	3.9 ± 2.5†	209.7 ± 27.3†	207.3 ± 26.1†
SnPP	17.4 ± 2.8§	342.7 ± 22.9‡	324.1 ± 21.4‡
CoPP	9.9 ± 3.1§	203.6 ± 31.1†	193.2 ± 31.5†

[a] THP-1 myelomonocytic cells (10^6/ml) were cultured in RPMI 1640 medium containing 10% heat-inactivated FCS, supplemented with medium, 25 μM (final concentration) iron, heme, ZnMP, ZnDP, PP, SnPP, or CoPP, and further treated with medium or 300 units/ml of hrIFN-γ. MHC class II antigen expression was quantified by immunofluorescence techniques as described in the Materials and Methods section. Data are presented as mean channel log fluorescence minus fluorescence of the isotope and the reagent control for three different experiments performed in duplicate (mean ± SE); $^{*}P < 0.001$; $\dagger P < 0.02$; $\ddagger P < 0.05$; $\S P > 0.05$, not significant (with respect to medium alone).

rise in antigen expression was observed in the presence of all porphyrins, heme, iron, and the control. Results are presented here as the mean of differences of log fluorescence intensities which indicate the degree of IFN-γ-induced HLA-DR expression. Note that after IFN-γ exposure, antigen expression was reduced when cells were exposed to heme, ZnMP, ZnDP, PP, or CoPP. The suppression of HLA-DR antigen expression was most pronounced when cells were exposed to iron or heme (Table I and Fig. 3). It can be seen in Figure 3 that there was a greater reduction in fluorescence intensity when heme was added to IFN-γ-stimulated cells as compared with unstimulated control cells. In contrast, IFN-γ-mediated HLA-DR expression was enhanced when cells were incubated with SnPP (Table I).

Discussion

Data presented here demonstrate that heme and several metalloporphyrins differently influence IFN-γ-mediated pathways in the human monocytic cell line, THP-1. In particular, these effects are seen on IFN-γ-induced neopterin formation, tryptophan degradation, and MHC class II antigen expression. Heme and several porphyrins, such as ZnDP and CoPP, reduced neopterin synthesis and decreased tryptophan degradation and kynurenine formation, as well as IFN-γ-induced MHC class II antigen expression. It is remarkable that ZnMP almost fully inhibits indoleamine 2,3-dioxygenase activity, whereas neopterin formation and MHC class II expression are only slightly reduced. The reasons for this are not clear; however, indoleamine 2,3-dioxygenase is a heme protein (32) that is able to bind a large number of different ligands (33).

Iron is an important component of heme, and results presented here show that iron is a potent inhibitor of the IFN-γ-mediated effects. It was shown previously that the inhibition of IFN-γ effects is not due to the interference with the key enzymes of IFN-γ-mediated pathways such as TLP-cyclohydrolase I (25). Therefore, the effects described above may possibly be due to interference of these substances with the IFN-γ signal itself.

Furthermore, PP did not produce significant differences in the IFN-γ effects as compared with the

control. This suggests that a metal ion, such as zinc, iron, or cobalt, may be necessary in the porphyrin ring in order to be effective. In contrast, concomitant addition of SnPP and IFN-γ to THP-1 cells slightly enhanced the IFN-γ effects. This was seen by increased neopterin synthesis, increased degradation of tryptophan and formation of kynurenine, and elevated MHC class II antigen expression. Novogrodsky *et al.* (22) observed that metalloporphyrins induce mitogenicity in peripheral T cells as tested by [^{3}H]thymidine uptake. Their results demonstrated that SnPP was more effective than heme or CoPP. Addition of interleukin 2 or the presence of macrophages enhanced this effect. Besides many other actions (34), IFN-γ is known to promote growth of T lymphocytes (35). Based upon our results, we suggest that the increased mitogenicity of SnPP toward T cells as compared with heme or CoPP is due primarily to an increase in IFN-γ signal efficiency by SnPP.

It is significant that SnPP is a potent competitive inhibitor of heme oxygenase activity (15). Furthermore, heme, iron, and several metalloporphyrins are known to induce heme oxygenase mRNA expression (20). Levels of heme oxygenase may affect many processes, and inhibition of heme oxygenase by SnPP could affect these processes. It should be noted that ZnPP and CoPP can also compete for heme oxygenase activity, but to a much lesser degree than that for SnPP (17). Since heme and SnPP have such contrasting effects on neopterin and HLA-DR expression, the levels of heme oxygenase activity may be important. In this respect, inhibition of heme oxygenase by SnPP will ultimately prevent increased levels of iron released from heme.

Others have shown (36) that metalloporphyrins differently influence ferrous iron and ascorbic acid-stimulated lipid peroxidation. Metalloporphyrins, which reduce the efficiency of the IFN-γ signal, such as heme or ZnPP, almost fully inhibit this type of lipid peroxidation. On the other hand, CoPP had only a slight effect on lipid peroxidation, while SnPP also increased IFN-γ activity and slightly enhanced lipid peroxidation. Therefore, it appears that the ability of metalloporphyrins to induce lipid peroxidation is, to a large extent, in parallel with their efficiency in modulating IFN-γ-mediated effects. Porphyrins that reduce lipid peroxidation also decrease the efficiency of the IFN-γ signal and vice versa.

Interferons have been demonstrated to suppress human erythropoiesis *in vitro* (37), and it was recently shown that patients suffering from HIV infection have elevated amounts of neopterin (17). The elevated levels of neopterin were ascribed to IFN-γ stimulation of patients' macrophages (7). Additionally, in some instances, adherent cells from patients with HIV infection have elevated levels of heme oxygenase (38). Thus, it appears that fluctuations in the levels of IFN, heme, porphyrin, iron, and heme oxygenase may also be associated with altered neopterin production. Based on previous observations and our present results, we conclude that some of the effects described for heme and metalloporphyrins may be due to the modulation of IFN-γ activity or efficiency of the IFN signal.

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