IMMUNE RESPONSE–ASSOCIATED PRODUCTION OF NEOPTERIN

Release from Macrophages Primarily under Control of Interferon-Gamma

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Neopterin is a pyrazino-pyrimidine compound derived from GTP. It represents an intermediate product in the synthetic pathway of biopterin. Biopterin itself is known to serve as an essential cofactor in neurotransmitter synthesis (1, 2). We have recently observed that immune responses in vitro and in vivo are invariably accompanied by increased neopterin levels. In vitro release of neopterin from human peripheral blood mononuclear cells (pbmnc)[1] was induced by stimulation with alloantigens, virally or chemically modified autologous cells or with mitogenic lectins (3). In vivo–increased neopterin excretion was demonstrated in patients suffering from infections with viruses (4) and intracellular bacterial or protozoal pathogens (5, 6), from rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (7) and from acute cellular graft rejection or graft-vs.-host disease (3, 8, 9). Elevated neopterin levels were also seen in patients with acquired immunodeficiency syndrome (AIDS) (10) and in certain tumor states (4). In these diseases an increase of neopterin excretion usually precedes the clinical manifestation and is correlated with the activity of the underlying disease. This behavior qualified neopterin as a useful tool for the biochemical monitoring of certain diseases. In fact, daily evaluation of neopterin excretion is now an essential component of the clinical monitoring of allograft recipients in several institutions. This study aimed to elucidate the cellular basis of the immune response–associated neopterin release in vitro. We present evidence that upon stimulation with factors derived from activated T cells human monocytes and macrophages (Mϕ) release neopterin into culture supernatants

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[1] *Abbreviations used in this paper:* AIDS, acquired immunodeficiency syndrome; Con A, concanavalin A; HPLC, high performance liquid chromatography; hur IFN$\gamma(\alpha_2)$, human recombinant interferon-gamma(alpha$_2$); LCL, lymphoblastoid cell lines; LGL, large granular lymphocytes; LPS, lipolysaccharide; Mϕ, macrophages; pbmnc, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

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and that interferon-gamma is the most active principle inducing this phenomenon.

Material and Methods

Reagents. Medium RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 20 mM of Hepes buffer (Gibco Laboratories, Grand Island, NY), antibiotics, and 15% heat-inactivated (56°C, 60 min) male pool serum was used throughout this study. Human nonrecombinant interferons α, β, and γ were obtained from Interferon Sciences and HEM Research (York Biological Intern., NJ) and from Virogen, Basel, Switzerland. Human recombinant interferons α_2 and γ as well as neutralizing monoclonal antibodies were obtained from Bender & Co. Vienna, Austria.

Mitogenic lectins phytohemagglutin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM) as well as lipopolysaccharide (LPS) were from Pharmacia, Uppsala, Sweden. Zymosan beads were obtained from Sigma Chemical Co. (St. Louis, MO) and were incubated at a concentration of 1 mg/ml with fresh human serum for 60 min at 37°C before use.

Cell Separation Techniques. Pbmnc were separated on Lymphoprep (Nygaard, Oslo, Norway). T cells and monocytes were isolated from heparinized venous blood of healthy volunteers by the combined use of Percoll gradient centrifugation and passage over Sephadex G10 (Pharmacia, Uppsala, Sweden) columns or adherence on glass petri dishes as previously described (11). B cells were obtained from single cell suspensions of human tonsils by negative selection with the pan-T cell monoclonal antibody T 101 and rabbit complement. Large granular lymphocytes (LGL) were enriched on Percoll density gradients as previously published (12). Purity of all cell populations except for LGL exceeded 95% and for LGL, 75%.

Cell Cultivation Techniques. 1×10^6 pbmnc, T cells, B cells, and LGL as well as 2.5×10^5 monocytes were cultured in 2.0 ml complete tissue culture medium in Costar macroplates (Costar, Cambridge, MA). They were stimulated for various times with mitogenic lectins, allogeneic pbmnc (1×10^6 cells) or lymphoblastoid cell lines (LCL) (2×10^5 cells), K 562 tumor cells (2×10^5 cells), and with LPS (10 μg/ml) and complement-coated zymosan beads. Supernatants were harvested and were assessed in parallel studies for their neopterin content with high performance liquid chromatography (HPLC) and for their capacity to induce neopterin release from unstimulated cells.

Measurement of Neopterin in Culture Supernatants. Neopterin contents were measured from culture supernatants by means of HPLC according to our previously published method (13) using an automatized system device (Neopterin System, Varian Intern., Zug, Switzerland).

Results

Neopterin Is Released from Human Mϕ and Monocytes upon Stimulation with Factors Derived from Activated T Cells. We have previously shown that human pbmnc upon stimulation release considerable amounts of neopterin into culture supernatants (reference 3, see also Table I). Further experiments with adherent cell-depleted responder lymphocytes at that time suggested that T cells might be responsible for this phenomenon (3). However, if T cells, rigorously depleted of monocytes by combined use of Percoll density fractionation and passage over Sephadex G10 columns or by cloning and expansion in IL-2, were tested, no more neopterin was detected (Table I). Seemingly, B cells, suspensions enriched for LGL or monocytes, and Mϕ did not release measurable amounts of neopterin (Table I). Macrophages stimulated with supernatants from activated but not from resting T cells demonstrated strong neopterin production, which on a per cell basis was approximately five times that of unseparated pbmnc (Table I). Mϕ

TABLE I

Neopterin Is Released from Macrophages upon Stimulation with Factors Derived from Activated T Cells

Cell type*	Activated with:	No. of experiments	Neopterin release‡
pbmnc	ϕ	29	<1
	PHA (1 μg/ml), Con A (10 μg/ml)	26	10 ± 2
	Allogeneic cells (LCL or pbmnc)	84	23 ± 5
T cells	ϕ	11	<1
	PHA (1 μg/ml), Con A (10 μg/ml)	4	<1
	Allogeneic cells (LCL)	16	<1
	Macrophage supernatants	3	<1
B cells	ϕ	3	<1
	PWM (25 μg/ml) and irradiated T cells	3	<1
LGL	ϕ	3	<1
	K 562 cells	3	<1
Mϕ	ϕ	31	<1
	PHA (1 μg/ml), Con A (10 μg/ml)	7	<1
	Allogeneic cells (LCL)	14	<1
	LPS (10 μg/ml)	1	<1
	Zymosan, C'coated (1 mg/ml)	3	<1
	Supernatants from resting T cells (25%)	14	<1
	Supernatants from alloreactive T cell clones, not restimulated (12.5%)	7	<1
	Supernatants from alloantigen-activated T cells (25%)	27	22 ± 2
	Supernatants from alloreactive T cell clones, restimulated (12.5%)	17	10 ± 2§
	Supernatants from PHA or Con A re-stimulated T cells (25%)	25	25 ± 2

* pbmnc, T cells, LGL, and Mϕ were isolated from the blood as described in Materials and Methods. B cells were obtained from tonsils by negative selection with anti-T cell antibodies and complement as also detailed in Materials and Methods. 1×10^6 pbmnc, B cells, T cells and LGL or 2.5×10^5 Mϕ were cultured in a total volume of 2.0 ml of complete medium.

‡ pmol/ml supernatant after 72–144 h of cultivation following activation, mean ± mes.

§ Only 5 of 17 clones tested produced detectable amounts of neopterin; data from these 5 clones are given in the table.

were approximately three times more effective as freshly isolated blood monocytes (three experiments, data not shown). A comparable release of neopterin was also seen from monoclonal Mϕ derived from a patient with well-differentiated monocytic leukemia (data not shown). Release of neopterin from activated Mϕ started after 24 h and was maximal after 2–3 d. Release of neopterin-inducing factors from T cells occurred within 24 h after stimulation, was resistant to irradiation of up to 2,000 rads, was optimal at a serum concentration of 1% in the culture, and could be completely blocked by addition by cyclosporin A at doses above 0.5 μg/ml; factors were nondialyzable and sensitive to treatment with pronase or acid at a pH of 2.0 (each observation is supported by at least three experiments, data not shown). As little as 1% of supernatant from activated T cells induced measurable release of neopterin from Mϕ and 25% was optimal in all 52 experiments performed.

Interferon-gamma Is the Active Principle in Supernatants of Activated T Cells Responsible for Induction of Neopterin Release from Mϕ. Data discussed in the previous paragraph indicated that only the activation of Mϕ with supernatants of transformed lymphocytes leads to release of neopterin. This observation and the blocking effect of cyclosporin A suggested that lymphokine(s) might represent the active principle(s) contained in these supernatants. First evidence for this

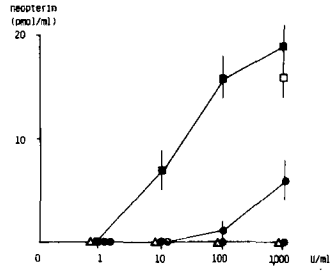

FIGURE 1. Induction of neopterin release from Mϕ by various human interferons. 2.5×10^5 Mϕ were cultured in 2.0 ml complete tissue culture medium in Costar macroplates. Various amounts of human interferons were added and the supernatants were harvested and assessed for neopterin content after 72 h. (⊙) nonrecombinant IFNα (7 experiments), (●) recombinant IFNα_2 (12 experiments), △ nonrecombinant IFNβ (14 experiments), □ nonrecombinant IFNγ (3 experiments), ■ recombinant IFNγ (18 experiments).

view was obtained in experiments in which various human interferons were directly added to Mϕ. Data are demonstrated in Fig. 1; they demonstrate that IFNγ is a most potent inducer of neopterin release. As little as 1 U hur IFNγ induced an already measurable amount of neopterin, whereas approximately 10^3 times higher concentrations of hur IFNα_2 were required for this effect. The capacity of the latter to induce neopterin release was also confirmed in in vivo studies: hur IFNα_2 in doses above 5×10^6 U within 24 h induced a sharp increase of neopterin excretion in 5 of 5 tumor patients studied (results not shown).

Maximal in vitro release of neopterin in the order of magnitude and with the kinetics of that induced by crude T cell supernatants was achieved by physiological doses of hur IFNγ in the range of 10^1 to 10^2 U/ml. Further experimental support for the role of IFNγ as the crucial component in T cell supernatants was obtained in neutralization studies involving acidification and specific antibodies. We first observed that acidification of T cell supernatants at pH values of 2.0 drastically reduced their capacity to induce neopterin release from Mϕ (three experiments, data not shown). Secondly, monoclonal antibodies against hur IFNγ but not against IFNα_2 or against IFN$\alpha_2\alpha_4$ were able to completely neutralize the induction of neopterin release and this neutralization could be overcome by re-addition of hur IFNγ. Data of one such experiment are demonstrated in Table II.

Discussion

Our original observation of elevated neopterin excretion in context with in vivo and in vitro immune stimulation prompted two different lines of further investigations: first, to assess the clinical utility of this biochemical marker in the monitoring of and the screening for certain diseases accompanied by hyperimmune stimulation; and secondly, the analysis of the cellular derivation and of the biological role of neopterin as well as of other components of the biopterin pathway. This second research topic gained further importance by the most promising results of our recent clinical trials, e.g. in the field of transplantation medicine or in the screening for patients affected with AIDS (8–10). In this article we present in vitro data on the cellular origin and the control of immune

TABLE II

Capacity of Supernatants from Activated T Cells to Induce Neopterin Release from Mϕ Is Completely Neutralized by Monoclonal Antibodies for IFNγ

Supernatants from:	Pretreated with:	Neopterin release from Mϕ* (pmol/ml)
Medium	None	<1
T cells stimulated with alloantigens	None	13
	mcab IFNα_2 25 μg/ml	14
	mcab IFN$\alpha_2\alpha_4$ 125 μg/ml	12
	mcab IFNγ 25 μg/ml	<1
T cells stimulated with PHA	None	11
	mcab IFNα_2 α_4 25 μg/ml	13
	mcab IFN$\alpha_2\alpha_4$ 125 μg/ml	11
	mcab IFNγ 25 μg/ml	<1

* Induced with 25% supernatant, for details see Legend of Table I.

response–associated elevation of neopterin. Results obtained with polyclonal and with monoclonal cellular reagents clearly demonstrate that monocytes and Mϕ are in fact capable of producing neopterin when stimulated with supernatants from activated T cells. In recent experiments performed in cooperation with Drs. Niederwieser and Curtius in Zurich, we could further support this new finding of the key role of Mϕ in the production of immune response–associated neopterin by simultaneous demonstration of the induction of a pteridine-specific GTP cyclohydrolase.[2] In the same study we further observed that Mϕ stimulated by T cell–derived molecules are capable of completing this metabolic pathway and synthesizing increased amounts of biopterin.[1] As far as the induction of neopterin release from Mϕ is concerned our in vitro data strongly point to the key role of interferon-gamma in the control of this phenomenon. This conclusion is supported by both the demonstration of neopterin release from Mϕ by addition of hur IFNγ and the complete neutralization of the inducing capacity of T cell supernatants by monoclonal antibodies specific for hur IFNγ. It also became apparent that at least IFNα_2 at much higher concentrations can induce neopterin release in vivo and in vitro. In this context it is of importance that high in vivo levels of acid-labile and thus IFNγ like interferon-α are observed in certain autoimmune diseases and in AIDS cases (14, 15). These states are also characterized by elevated neopterin excretion (7, 10). In conclusion, we obtained in vitro evidence that immune response–associated neopterin release is a consequence of the activation of the biopterin synthetic pathway in Mϕ and that this process is under control of lymphokines. This in vitro model is in keeping with the above-discussed clinical findings concerning in vivo interferon levels or neopterin excretion in relation to disease states, and with our previous observation that lethally irradiated patients during the time of hemolymphatic aplasia are unable to increase neopterin excretion in response to immune challenge (9). It also points to the fact that a metabolic pathway so far exclusively known to be involved in the generation of an essential cofactor of neurotransmitter synthesis is activated in bone marrow–derived cells during immune responses. Whether

[2] Niederwieser, A., G. Schödoon, H. C. Curtius, A. Lang, H. Wachter, Ch. Huber. Pteridine synthesis in mononuclear blood cells. Manuscript in preparation.

this represents a means by which immune cells communicate with each other or with the neuroendocrine system remains to be further investigated; in any case, recognition of signals emitted from this pathway in context with immune stimulation might gain clinically relevant information about certain aspects of the activation stage of the immune system itself.

Summary

Neopterin, a compound derived from GTP, represents a precursor molecule of biopterin that is an essential cofactor in neurotransmitter synthesis. We have recently reported that in vivo as well as in vitro immune responses are accompanied by an increased release of neopterin and that this phenomenon can be used for the biochemical monitoring of diseases accompanied by hyperimmune stimulation. This article deals with the cellular origin and the control of this immune response–associated neopterin release in vitro. Using highly purified or monoclonal cellular reagents we demonstrate that macrophages (Mϕ) stimulated with supernatants from activated T cells release large amounts of neopterin into culture supernatants. Further experiments involving induction of neopterin release from Mϕ with various human recombinant interferons (IFNs) or neutralization of the effect of T cell supernatants with various monoclonal anti-IFN antibodies revealed immune IFN as the active principle. It thus appears that a metabolic pathway so far exclusively known in context with the generation of an essential cofactor of neurotransmitter-synthesis during immune responses is also activated in Mϕ under stringent control by immune IFN-like lymphokines.

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