Vol. 132, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1985 Pages 404-411

SYNTHESIS AND INTERFERON-γ CONTROLLED RELEASE OF PTERIDINES DURING ACTIVATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

I. Ziegler

Institut für Toxikologie und Biochemie der Gesellschaft
für Strahlen-und Umweltforschung, Abt. Zellchemie,
Landwehrstr. 61, D-8000 München 2, Germany

Received September 6, 1985

Lectin stimulation of human peripheral blood mononuclear cells causes an increase in neopterin, biopterin, 6-hydroxymethylpterin and 6-formylpterin, as was determined by HPLC after iodine oxidation of the acid extract. After 72 h, pteridines peak at levels 5-10 fold as compared to resting cells. Levels decline to initial values during the following 24 h. Changes in pteridine proportions indicate that the synthesis of tetrahydrobiopterin proceeding from dihydroneopterin triphosphate is controlled during the process of lymphocyte activation. The release of both cellular neopterin and biopterin, but not of 6-hydroxymethylpterin and its aldehyde, is controlled by interferon-γ.

The screening of urinary neopterin and biopterin levels during neoplasia, viral infections, autoimmune diseases, and allograft rejection had indicated that the activation of T-lymphocytes is associated with the production of pteridines (1,2). Evidently, these pteridines are derived from the immunoproliferating cells. Macrophages were identified as the source of neopterin. Its release is triggered by IFN-γ [1] (3,4). Biopterin was shown to accumulate in the pbmc, e.g., during graft versus host reaction (5,6). In the first *in vitro* model the kinetics of pteridine accumulation in lectin stimulated mouse spleen lymphocytes were recorded (7). The formation of biopterin, 6-hydroxymethylpterin and 6-formylpterin is an early event and precedes the onset of DNA synthesis; neopterin is lacking in these cells of non-primate origin. The increase of GTP-cyclohydrolase activity in mononuclear blood cells analyzed five days after lectin stimulation (8) further indicates that pteridine biosynthesis takes place in these proliferating cells. In order to gain a closer

[1] Abbreviations: IFN-γ = interferon-γ ; pbmc = peripheral blood mononuclear cells; PHA = phythemagglutinin ; Con A = concanavalin A; PMA = phorbol 12-myristate 13-acetate; IL-2 = interleukin 2.

0006-291X/85 $1.50

understanding of the changes ocurring during stimulation of human pbmc the present investigations addressed the kinetics of pteridine formation after lectin stimulation of these cells.

Previous experiments had shown that some reduced pterins modulate the rate of [^{3}H] thymidine incorporation in lectin stimulated mouse spleen lymphocytes (9) and, particularly, the activity of IL-2 (10). A possible mediator function of pteridines depends upon their previous release from pteridine-synthesizing cells. Therefore, as a second topic the role of IFN-γ in controlling the release of pteridines from human pbmc was included in our studies.

MATERIALS AND METHODS

Stimulation and cultivation of pbmc. Cells were isolated from heparin anticoagulated blood by the Ficoll-Hypaque centrifugation method (11). Lectin stimulation was started with 40-60 ml batches in Falcon flasks containing 7.5 x 10^5 cells/ml. Medium composition and culture conditions are described in (9). Optimum activation was achieved at concentrations of 3-10 ug/ml with Con A, at 2-9 ug/ml with PHA dependent on the donor. The activation rates declined above and below these values. The data in Figs. 1 and 2 were obtained from 10 independent experiments.

Harvesting of the stimulated cells, incubation with IFN-γ and anti-IFN-γ. At the periods indicated [^{3}H]thymidine incorporation was determined in 200 ul aliquots as described in (9). The cultures were labelled with 1 uCi/well (0.4 Ci/mmol). After determination of viable cell counts, 6-8 ml aliquots were used for pteridine analysis. The centrifuged and washed cells were kept at -20°C. In separate experiments cells were resuspended in phosphate buffered saline (Dulbecco; lacking Ca^{++} and Mg^{++}), at a density of 1 x 10^6 cells/ml and incubated with IFN-γ , (from PMA stimulated pbmc; Fa. Biotest, Frankfurt), and with polyclonal anti-IFN-γ(Fa.Paesel, Frankfurt), respectively. Subsequently, cells were separated by centrifugation. The supernatants were lyophilized and both fractions were stored at -20°C. The data in Fig. 3 were obtained from 6 independent experiments.

Identification and quantitative determination of pteridines by reverse phase HPLC. The method is described in (7). Briefly, the oxidized pteridines were pre-purified by Dowex H^+ ion exchange chromatography, separated by reverse-phase HPLC (H_3PO_4(3 mM)/7% (v/v) methanol/1% (v/v) acetonitrile as mobile phase) , and monitored by their fluorescence at 450 nm (excitation wavelength 350 nm).

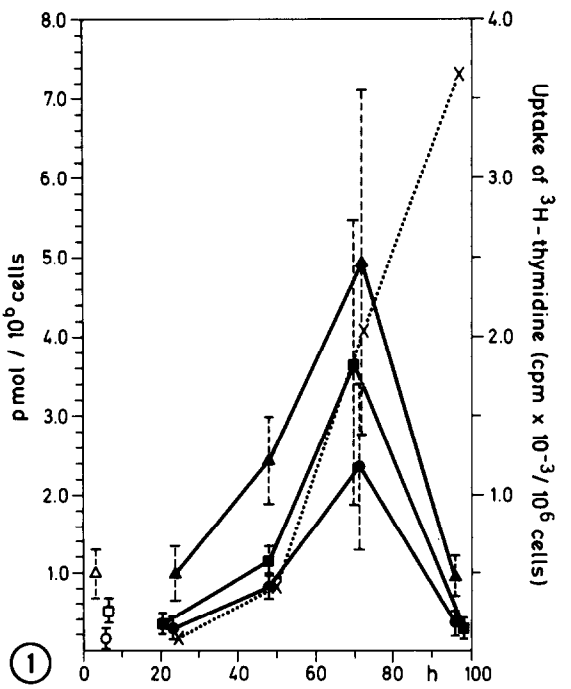
RESULTS

Kinetics of pteridine formation during lectin activation. A typical HPLC elution profile of pteridines from activated mouse spleen lymphocytes is presented in (7). The murine pteridines, biopterin, 6-hydroxymethylpterin and 6-formylpterin, were also identified in human pbmc. Again, the latter pteri-

dine composed about 15 % of the carbinol and for reasons already described in (7), both the aldehyde and the carbinol were considered jointly in the data given below. In addition to the murine pteridines human pbmc are characterized by neopterin.

The pteridine concentrations of resting cells, kept in medium remain approximately at the same low levels which are depicted in Fig. 1 for time zero. After a period of 48h they finally fall, probably due to decay. Lectin stimulation of the pbmc causes a marked increase in all pteridine fractions. Their levels reach a maximum at 72 h and drop to the initial values within the subsequent further 24 h. Thus they increase, peak, and decline earlier than DNA synthesis.

A closer data analysis reveals that the relative amounts of the three pteridine fractions are changing during the course of the activation period. Fig. 2 shows their of the percentage distribution. Neopterin comprises about



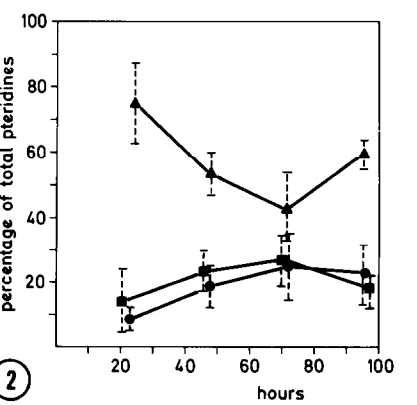


Fig.1. Pteridine levels and DNA synthesis in lectin stimulated human pbmc. ▲——▲ neopterin; ●——● biopterin; ■——■ 6-hydroxymethylpterin + 6-formylpterin. X·····X [^{3}H]thymidine incorporation. ⊥ S.D.

Fig.2. Proportions of pteridines during lectin stimulation of human pbmc. ▲——▲ neopterin; ●——● biopterin; ■——■ 6-hydroxymethylpterin + 6-formylpterin. ⊥ S.D.

80% during the first period of activation but drops to nearly half of this value in the following period. Concomitantly, the proportion of the other pteridines, especially of biopterin, is increasing markedly. In each of the experiments, lowest percentage contribution of neopterin coincided with the climax of pteridine accumulation after 72 h.

Control of pteridine release by IFN-γ . Initially, attempts were made to quantitate the pteridines in the culture medium to which IFN-γ and anti-IFN-γ, respectively had been added. However, RPMI 1640 medium contains gross amounts of interfering fluorescent compounds which could not be eliminated by the pre-purifaction procedure. Moreover, fetal calf serum itself introduces high levels of biopterin (7). Therefore, this allows only a rough estimation of pteridine release from the activated cells into the medium. Pilot tests indicated that the exposure of activated cells to phosphate buffered saline, containing IFN-γ (100 U/ml) and anti-IFN-γ (0.02-1.0 ug/ml), respectively, during a period of 3 h yielded results comparable to those obtained with the multicomponent standard medium. Moreover, the use of phosphate buffered saline yielded clear separation and quantitative determination of the pteridines by HPLC, provided the supernatants were processed in the same way as the cells (7).

Fig. 3 shows the action of IFN-γ on the release of pteridines from the activated cells. Case "a" of each panel depicts the pteridine levels of the freshly harvested cells. Cases "b" illustrate the amounts of cellular and of supernatant pteridines after incubation with IFN-γ . Cases "c" show their levels in the absence of IFN-γ ; additionally the endogenous lymphokine was neutralized by anti-IFN-γ. It is evident that substantial amounts of neopterin (left panel) and biopterin (central panel) are released by the addition of IFN-γ . Its antibody abrogates this effect. It is calculated that under our experimental conditions the IFN-γ induced release renders the medium 2-8 $\times 10^{-9}$ M with respect to both pteridines. Their cellular levels are not reduced after this period. It is therefore concluded that new pteridine synthesis had occurred during exposure of the cells to IFN-γ.

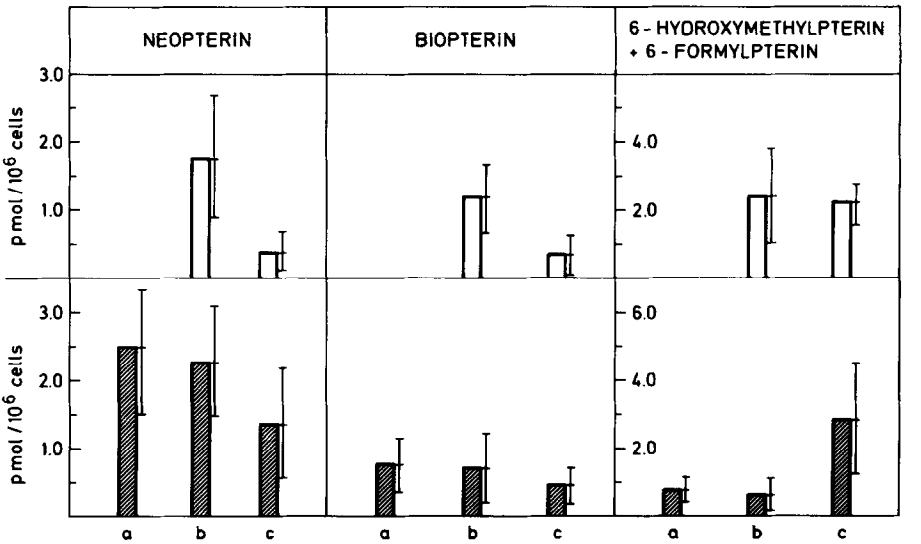


Fig.3. Action of IFN-γ on pteridine release from lectin stimulated human pbmc. a = cells after harvesting from the medium; b = incubation with IFN-γ for 3 h; c = incubation with anti IFN-γ for 3 h. For details see Materials and Methods, and Results.
▨ cellular pteridines; ⊓ pteridines from supernatant; ⊢ S.D.

Contrarily, addition of IFN-γ as well as the antibody to phosphate buffered saline causes a release of 6-hydroxymethylpterin and its aldehyde. Therefore it is concluded that IFN-γ is not responsible for the release of these pteridines. The data indicates that the elimination of this lymphokine may possibly cause an increase in the cellular levels of these pteridines.

The short-term IFN-γ -induced release of biopterin and neopterin described above occurs only in activated pbmc, but not in resting cells which had been kept in medium for periods of 2-4 days. After their exposure to phosphate buffered saline or to IFN-γ, a fraction of the pteridines is transported into the supernatant but the total amounts remain at the same low levels as described above.

DISCUSSION

These experiments demonstrate the kinetics of pteridine accumulation during activation of human pbmc for the first time. The inrease, culmination and decline of their pteridine levels precede the respective phases of DNA synthesis. Samples collected after an activation period of only five days may

explain the low biopterin levels reported elsewhere (8). Our data show that the pteridine formation in human pbmc coincides with the period of their IL-2 production (cf. 12). This provides the basis for the modulating effect of tetrahydrobiopterin on the activity of this lymphokine (10), finally resulting in the enhancement of lymphocyte proliferation (9). Neopterin and dihydroneopterin were shown to lack such an effect (9).

A positive modulation was found to be dependent on a subtle interrelationship of both the lymphokine and the tetrahydrobiopterin concentrations (9,10). Therefore, control of pteridine biosythesis and release is an essential prerequisite for achieving optimum concentration. First indications for such control steps are obtained. Pbmc activation entails increased activity of GTP cyclohydrolase activity (8) and thus initiates increased production of dihydroneopterin triphosphate; it still remains undetermined whether this is due to an enzyme activation or to enzyme synthesis. After elimination of the triphosphate group dihydroneopterin may either proceed to tetrahydrobiopterin (cf. 13) or be released in the reduced (14) or in the oxidized form (1), respectively. Elimination of excess pteridine may occur at this step. During further progress of tetrahydrobiopterin synthesis, sepiapterin reductase is presumed to catalyze the reduction of the subsequent intermediate 6-pyruvoyl-tetrahydropterin (cf. 13). The data presented above, indicate an acceleration of this pathway during the first 72 h in that increasing proportions of dihydroneopterin convert to tetrahydrobiopterin. Information on regulatory properties of sepiapterin reductase is needed to explain a further possible step of control.

IFN-γ plays a regulatory role in that both neopterin (3,4) and biopterin release is triggered by this lymphokine. Additionally, increases in macrophage GTP cyclohydrolase activity and in intracellular neopterin levels were ascribed to the action of IFN-γ (15). The mitogen induced increase in cellular pteridines may possibly operate via IFN-γ which is produced by the activated T cells (cf. 12). At present, a more immediate action of lectins can neither be substantiated nor be excluded. Feedback inhibition by tetrahydro-

biopterin is reported for GTP cyclohydrolase from rat tissues (16). Therefore, IFN- γ induced release of this pteridine should eliminate its effect. This may contribute to the increase in total (intracellular + released) amounts of pteridines and may cooperate with a more immediate activation of pteridine synthesis by lectins.

In contrast to biopterin and neopterin, the release of 6-hydroxymethylpterin and its aldehyde is not controlled by IFN-γ. Their distinct characteristics and possible, synthetic pathway is discussed in (7) and is currently under study. Using L1210 mouse leukemia cells it was shown that transport of 6-hydroxymethylpterin is energy-dependent (17). Additional experiments have to decide whether the efflux of the carbinol and its aldehyde is only dependent on concentration gradients or whether it is controlled by other than IFN-γ lymphokines.

ACKNOWLEDGEMENTS

The work was supported by the Deutsche Forschungsgemeinschaft and by the Bundesministerium für Forschung und Technologie. We thank the Blutspendezentrale des Bayerischen Roten Kreuzes, and all volunteers for cooperation. The experiments were skillfully assisted by C.Altmann.

REFERENCES

1. Fuchs,D., Hausen,A., Huber,Ch., Margreiter,R., Reibnegger,G., Spielberger,M. and Wachter,H. (1982) H.S.Zeitschr.Physiol.Chem. 363, 661-664.

2. Rokos,H., Rokos,K., Frisius,H., Kirstaedter,H.J.(1980) Clin.Chim.Acta 105, 275-286.

3. Huber,Ch., Fuchs,D., Hausen,A., Margreiter,R., Reibnegger,G., Spielberger,M., and Wachter,H. (1983) J.Immunol. 130, 1047-1050.

4. Huber,Ch., Batchelor,J.R., Fuchs,D., Hausen,A., Lang,A., Niederwieser, D., Reibnegger,G., Swetley,P., Troppmair,J., and Wachter,H. (1984) J. Exp.Med. 160, 310 - 316.

5. Fink.M., Jehn,W., Wilmanns,W., Rokos,H., and Ziegler,I. (1983) in: Biochemical and Clinical Aspects of Pteridines. Eds.H.Ch. Curtius, W.Pfleiderer, H.Wachter, W.de Gruyter, Berlin, New York, pp.223-233.

6. Fink,M., Ziegler,I., and Rokos, H.. (1985) in: Biochemical and Clinical Aspects of Pteridines. Eds.H.Wachter, W.Pfleiderer, H.Ch.Curtius. W.de Gruyter, Berlin, New York (in press).

7. Ziegler,I. (1985) J.Cell.Biochem. 28, 197-206.

8. Blau,N., Joller,P., Atarés,M., Cardesa-Garcia,J., and Niederwieser,A., (1985) Clin.Chim.Acta 148, 47-52.

9. Ziegler,I., Hamm,U., Berndt,J. (1983) Cancer Res.43, 5356-5359.

10. Ziegler,I., Schwuléra,U., Sonneborn,H.-H., Müller,W.J.P. (1985) Naturwiss. 72, 330-331.

11. Böyum,A. (1968) Scand.J.Clin.Lab.Invest. 21 (Suppl.97) 77-89.

12. Farrar,J.J., Benjamin,W.R., Hilfiker,M.L., Howard,M., Farrar,W.L., and Fuller-Farrar,J. (1982) Immunol.Rev. 63, 129-166.

13. Nichol,A., Smith,G.K., and Duch D.S. (1985) Ann.Rev.Biochem. 54, 729-764.

14. Levine,R.A., and Milstien,Sh. (1984) in: Biochemical and Clinical Aspects of Pteridines. Eds.W.Pfleiderer, H.Wachter, and H.Ch.Curtius. W.de Gruyter, Berlin, New York, pp.277-284.

15. Troppmair,J., Lang,A., Huber,Ch., Schoedon,G., Niederwieser,A., and Adolf,G. (1985) in: Biochemical and Clinical Aspects of Pteridines. Eds. H.Wachter, W.Pfleiderer, H.Ch.Curtius, W.de Gruyter, Berlin, New York (in press).

16. Bellahsene,Z., Dhondt,J.L., and Farriaux,J.P. (1984) Biochem.J. 217, 59-65.

17. Suresh, M.R. and Huennekens, F.M. (1982) Biochem. Internat. 4, 533-541.