EFFECT OF NICOTINAMIDE ON RNA AND DNA SYNTHESIS AND ON POLY(ADP-RIBOSE) POLYMERASE ACTIVITY IN NORMAL AND PHYTOHEMAGGLUTININ STIMULATED HUMAN LYMPHOCYTES

C. ROCHETTE-EGLY+, M. E. ITTEL*, J. BILEN and P. MANDEL
Centre de Neurochimie du CNRS, 11, rue Humann, 67085 Strasbourg Cedex, France

Received 5 August 1980
Revised version received 3 September 1980

1. Introduction

The nuclei of eukaryotic cells contain the enzyme poly(ADP-ribose) polymerase which converts NAD into poly(ADP-ribose) with the elimination of nicotinamide [1,2].

Several reports suggest that poly(ADP-ribose) polymerase is involved in the regulation of eukaryotic DNA synthesis and in the DNA repair mechanisms (reviewed [3–5]). A higher poly(ADP-ribose) polymerase activity in leukemic leukocytes than in lymphocytes from normal donors was observed [6,7]. Similarly, in human and pig lymphocytes, the activity of poly(ADP-ribose) polymerase increased together with DNA synthesis, induced by phytohemagglutinin PHA [7,8]. Finally, an increase in poly(ADP-ribose) polymerase activity in parallel to cellular NAD decrease after treatment of cells with DNA-damaging agents was reported [9,10]. It is of interest to define whether the well-known decrease in NAD content of proliferating tumoral cells is also correlated with an increase in poly(ADP-ribose) polymerase activity and whether changes in poly(ADP-ribose) polymerase activity correlate with cell proliferation. Nicotinamide at high concentrations, is a well-known competitive inhibitor of poly(ADP-ribose) polymerase [11,12]. This study was undertaken in order to determine whether nicotinamide added at various concentrations to the culture medium of PHA-stimulated human lymphocytes will affect poly(ADP-ribose) polymerase activity, DNA synthesis as well as cell proliferation.

Abbreviations: ADP-ribose, adenosine 5′-diphosphate ribose; poly(ADP-ribose), polymer of adenosine 5′-diphosphate ribose; NAD, nicotinamide adenine dinucleotide

+ Present address: Unité 61 de l'INSERM, 3, Avenue Molière, 67200 Strasbourg, France

* To whom correspondance should be addressed

2. Materials and methods

Lymphoprep was obtained from Nyegard (Oslo). Purified phytohemagglutinin HA16 (PHA) was purchased from Wellcome Research (Beckenham, Kent) and nicotinamide from Merck (Darmstadt). Eagle's basal medium from Eurobio (France) and fetal calf serum were obtained from Gibco (Paisley). [^{14}C]-Thymidine (spec. act. 53 mCi/mmol), [^{3}H]uridine (spec. act. 10 Ci/mmol), [*adenine* 2,8-^{3}H]NAD^{+} (spec. act. 2.96 Ci/mmol), Protosol, Econofluor and Omnifluor were purchased from New England Nuclear (Boston, MA). Non-labeled NAD^{+} was from Sigma Chemical Co. (St Louis, MO). All other chemicals were of analytical grade and were purchased either from Prolabo (France) or from Merck (Darmstadt).

2.1. *Cell preparation*

Human peripheral small lymphocytes were isolated from blood of normal donors by density gradient centrifugation using lymphoprep. The method used was slightly modified from [13]. Briefly: 5 ml blood, diluted with an equal volume of PBS (saline phosphate buffer, pH 7) were layered onto 4 ml lymphoprep in a 16 × 125 mm plastic culture tube (Corning). The separation of cells was achieved by centrifugation for 15 min at 400 × *g*. The lymphocytes were obtained from the plasma/lymphoprep interface and washed

with PBS. In this preparation the lymphocytes were recovered in 90–100% yield, constituted 95–98% of the cells, and were 98% viabilety as shown by the trypan blue-exclusion test.

2.2. *Evaluation of RNA and DNA synthesis*

Viable cells (5×10^5) were resuspended in 16 × 125 mm plastic culture tubes (Corning) containing 5 ml Eagle's minimum medium supplemented with 20% fetal calf serum. After addition of 1 μg/ml phytohemagglutinin, the cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, in the absence or presence of different concentrations of nicotinamide. At appropriate time intervals after the addition of the mitogen (6, 10, 24, 30, 48, 60, 72 and 96 h), cells were assayed for DNA and RNA synthesis by a 1 h pulse with [^{14}C]thymidine (0.1 μCi/ml) and [^{3}H]uridine (1 μCi/ml), respectively. The cells were centrifuged, washed with PBS, precipitated with ice-cold 10% trichloroacetic acid and collected on glass fiber filters (GF/C, Whatman). All filters were washed with 15 ml trichloroacetic acid, transferred to scintillation vials and dissolved at 60°C in 0.5 ml Protosol; 10 ml of Econofluor were added and the radioactivity measured in a scintillation spectrometer. For control experiments, the incorporation of [^{14}C]thymidine and [^{3}H]uridine was determined in parallel in unstimulated cultures with or without nicotinamide. Results are expressed as cpm . h^{-1} . 10^6 viable cells^{-1}.

2.3. *Assays of poly(ADP-ribose) polymerase*

Cell pellets were suspended at 2×10^8 cells/ml and treated in a permeabilizing buffer as in [14]. Poly(ADP-ribose) polymerase activity was measured by the incorporation of radioactivity into acid insoluble material using [*adenine*-H^3]NAD$^+$ as substrate. The incubation mixture contained 0.1 M Tris–HCl buffer (pH 7.9), 8 mM $MgCl_2$, 0.4 mM dithiothreitol, 20% glycerol, 30 μl inactivated calf thymus chromatin solution (7 μg DNA, 13 μg protein) [15], 10 nmol [*adenine*-^{3}H]NAD$^+$ (50 000 cpm) and 2×10^6 permeabilized cells in 135 μl final vol. The incubation was performed at 25°C for 5 min and was stopped by addition of 2 ml 10% trichloroacetic acid containing 0.02 M sodium pyrophosphate and 0.1 ml 1% bovine serum albumin. After 30 min at 0°C, the precipitate was collected on a Whatman GF/B glass fiber paper and washed 4 times with 10 ml ice-cold 5% trichloroacetic acid containing 0.02 M sodium pyrophosphate, and once with 4 ml ethanol–ether (1:1, v/v) solution. After drying (30 min, 100°C) the filter was counted in a Intertechnique spectrometer SL 40. The scintillation fluid used was 10 ml 0.4% Omnifluor in toluene. Under our experimental conditions the counting efficiency was constant (26%). In all cases activities were measured under conditions of proportionality of enzyme activity with time or enzyme protein. The enzyme assays were always performed in triplicate. Results are given as mean ± SD. One unit of enzyme was defined as the amount of 1 pmol [*adenine*-^{3}H]-ADP-ribose incorporated into acid insoluble product per 5 min at 25°C under the described conditions. The specific activity is expressed as units per 10^6 cells.

3. Results

Under our culture conditions human lymphoid cells respond optimally to 1 μg PHA/ml as tested by DNA synthesis (fig.1). After the addition of the

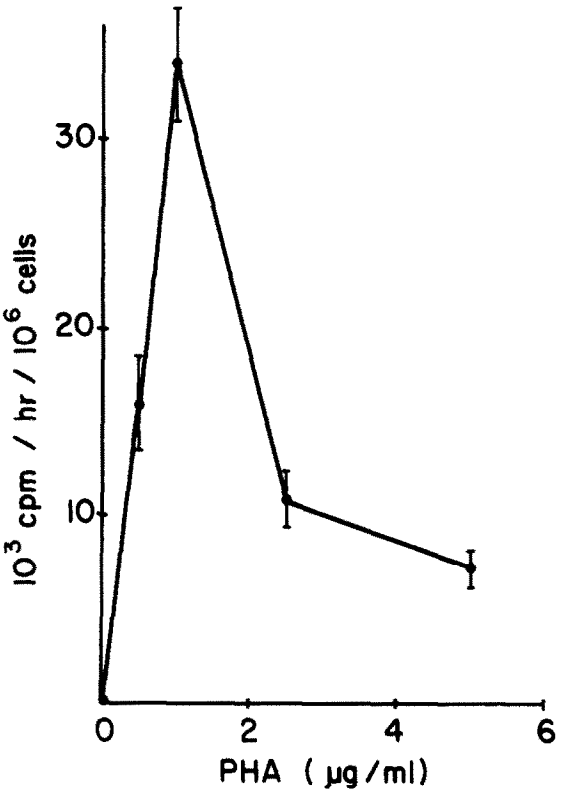

Fig.1. [^{3}H]Thymidine incorporation in human lymphocytes treated with increasing doses of PHA. DNA synthesis was determined 72 h after the addition of the mitogen. Results are given as the mean ± SE of 4 expt.

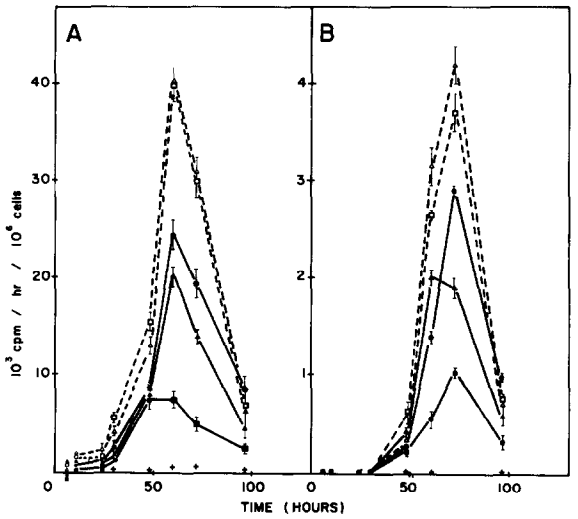

Fig.2. Effect of nicotinamide on [^{3}H]uridine (A) and [^{14}C]-thymidine (B) incorporation rates. Control (+——+); in 1 μg/ml PHA-treated lymphocytes in the absence (●——●) or in the presence of nicotinamide at different concentrations: 0.1 mM (△——△), 0.5 mM (□——□), 5 mM (▲——▲) and 10 mM (■——■). At appropriate intervals, cells were assayed for RNA and DNA synthesis. Results are given as the mean ± SE of 4 expt.

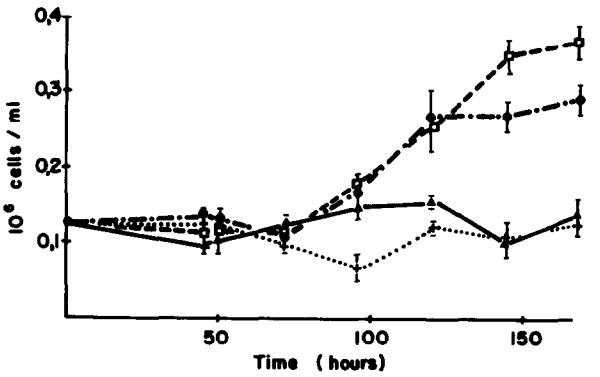

Fig.3. Effect of nicotinamide on the doubling of PHA-stimulated lymphocytes. Lymphocytes were cultured with no addition (+——+), 1 μg PHA/ml in the absence (●——●) or presence of 0.1 mM (□——□) or 10 mM (▲——▲) nicotinamide. At appropriate intervals, cells were counted and their viability evaluated by the trypan blue-exclusion test. Results expressed as 10^6 cells/ml are the mean ± SE of 3 expt.

mitogen to human lymphocytes in culture medium (fig.2), RNA and DNA synthesis reach a maximum at 60 and 72 h, respectively and the number of cells doubles after 120 h (fig.3). Addition at the onset of the culture, of 0.1 and 0.5 mM nicotinamide to optimally PHA-stimulated lymphocytes, led to 60% and 40% enhancements of DNA synthesis at 72 h, respectively, as determined by [^{14}C]thymidine incorporation (fig.2B). However, when nicotinamide was increased up to 10 mM, a marked inhibition of the mitogen-induced DNA synthesis was noted, during all the cultivation period (fig.2B). Similar stimulatory and inhibitory effects of nicotinamide at low and high concentrations respectively, were also observed on RNA synthesis as determined by [^{3}H]uridine incorporation (fig.2A). Furthermore, the PHA-induced increase of the number of lymphocytes was enhanced by 0.1 mM nicotinamide, whereas it was abolished by 10 mM nicotinamide (fig.3).

Poly(ADP-ribose) polymerase activity was measured at 24, 48, 72 and 96 h of culture in control and PHA-stimulated cells cultured in the presence or absence of nicotinamide (table 1). In cells treated exclusively with PHA, there is an increase of poly-(ADP-ribose) polymerase activity at 48 h which is significantly higher at 72 h and decreases slightly at 96 h. When cultured in the presence of PHA and 0.1 mM nicotinamide, there are no significant differences in poly(ADP-ribose) polymerase activity of PHA-treated cells, except at 96 h where the enzymatic activity is slightly higher. Finally, when cultured in the presence of PHA and 10 mM nicotinamide, the stimulation of the poly(ADP-ribose) polymerase activity by PHA is abolished, and the values are similar to the activity in control cells which were cultured with 10 mM nicotinamide in the absence of PHA.

It is noteworthy that poly(ADP-ribose) polymerase activity in control cells measured in our incubation conditions that is after removal of nicotinamide does not vary although high nicotinamide concentrations were present before in the culture medium. Moreover, nicotinamide present in the culture medium did not alter cell viability, as determined by the trypan blue-exclusion test, nor did it affect [^{14}C]thymidine and [^{3}H]uridine incorporation when added to the culture medium in the absence of PHA.

4. Discussion

When DNA synthesis in control and PHA-stimulated normal human lymphocytes is compared, a low level of thymidine incorporation is found in control cells, while PHA-stimulated cells demonstrate a characteristic increase in DNA and RNA synthesis at 72 and

Table 1
Effect of PHA and of nicotinamide on poly(ADP-ribose) polymerase activity in cultured human lymphocytes (ADPR incorporated in 10^6 PHA stimulated cells/ADPR incorporated in 10^6 control cells)

Nicotinamide in culture medium	Time of culture			
	24 h	48 h	72 h	96 h
–	0.84 ± 0.16 (n = 5)	2.34 ± 1.3[b] (n = 5)	5.17 ± 1.62[a,b] (n = 4)	4.37 ± 1.64[a] (n = 4)
0.1 mM	0.67 ± 0.27 (n = 4)	1.42 ± 0.44[b] (n = 4)	5.17 ± 1.87[a,b] (n = 4)	6.72 ± 1.02[a] (n = 4)
10 mM	1.15 ± 0.36 (n = 4)	0.97 ± 0.23 (n = 4)	1.5 ± 0.5[c] (n = 3)	1.96 ± 0.63[c] (n = 4)

[a] Corresponds to significant differences as evaluated by Student-Fisher test between PHA and control cells

[b] Corresponds to significant differences as evaluated by Student-Fisher test between 24 h and 48 h PHA-stimulated cells or between 48 h and 72 h PHA-stimulated cells

[c] Corresponds to significant differences as evaluated by Student-Fisher test between PHA- and PHA + nicotinamide-treated cells for a given time

Results are arithmetic mean ratios ± SD from n determinations

60 h, respectively and a subsequent decrease to almost initial values. In parallel the poly(ADP-ribose) polymerase activity increases strongly until 72 h and remains relatively high at 96 h.

In the presence of 0.1 mM nicotinamide there is no significant change in poly(ADP-ribose) polymerase activity, although at this concentration, nicotinamide produces an additional stimulation of both DNA and RNA synthesis. The absence of correlation between cellular DNA, RNA synthesis and poly(ADP-ribose) polymerase activity under the effect of 0.1 mM nicotinamide measured in cell homogenates does not exclude a change in the activity of the enzyme in vivo due to the well-known increase of the substrate NAD, by a low concentration of nicotinamide [16].

A high concentration of nicotinamide in culture medium reduces poly(ADP-ribose) polymerase activity of PHA-stimulated cells as well as cell proliferation and DNA synthesis. These results are in agreement with those in [17] where inhibition of poly(ADP-ribose) synthesis by 20 mM nicotinamide during the S-phase was accompanied by an inhibition of the DNA synthesis in synchronized mouse L-cells. Thus, it seems very likely that inhibition of poly(ADP-ribose) polymerase by a high concentration of nicotinamide is involved in the blockage of the response of lymphocytes to PHA.

Acknowledgements

The authors wish to thank Professor J. Kempf for his interest in this work and Mr J. L. Toussaint for his excellent technical assistance.

References

[1] Chambon, P., Weil, J. D., Doly, J., Strosser, M. T. and Mandel, P. (1966) Biochem. Biophys. Res. Commun. 25, 638–643.
[2] Fujimura, S., Hasegawa, S. and Sugimura, T. (1967) Biochim. Biophys. Acta 134, 496–499.
[3] Hayaishi, O. and Ueda, K. (1977) Annu. Rev. Biochem. 46, 95–116.
[4] Hilz, H. and Stone, P. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1–58.
[5] Purnell, M. R., Stone, P. R. and Whish, W. J. D. (1980) Biochem. Soc. Trans. 8, 215–227.
[6] Burzio, L., Reich, L. and Koide, S. S. (1975) Proc. Soc. Exp. Biol. Med. 149, 933–938.
[7] Berger, N. A., Adams, J. W., Sikorski, G. W., Petzold, S. J. and Shearer, W. T. (1978) J. Clin. Invest. 62, 111–118.
[8] Lehmann, A. R., Kirk-Bell, S., Shall, S. and Whish, W. J. D. (1974) Exp. Cell Res. 83, 63–72.
[9] Whish, W. J. D., Davies, M. I. and Shall, S. (1975) Biochem. Biophys. Res. Commun. 65, 722–730.

[10] Skidmore, C. J., Davies, M. I., Omidiji, O., Zia'ee, A. and Shall, S. (1979) in: Proc. 5th Int. Conf. Novel ADP-ribosylations of regulatory enzymes and proteins (Smulson, M. E. and Sugimura, T. eds) Elsevier/North-Holland, Amsterdam, New York.
[11] Fujimura, S., Hasegawa, S., Shimizu, Y. and Sugimura, T. (1967) Biochim. Biophys. Acta 145, 247–259.
[12] Niedergang, C., Okazaki, H. and Mandel, P. (1979) Eur. J. Biochem. 102, 43–57.
[13] Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21 suppl., 97.
[14] Berger, N. A., Weber, G. and Kaichi, A. S. (1978) Biochim. Biophys. Acta 519, 87–104.
[15] Marushige, K. and Bonner, J. (1966) J. Mol. Biol. 15, 160–174.
[16] Revel, M. and Mandel, P. (1962) Cancer Res. 22, 456–462.
[17] Colyer, R. A., Burdette, K. E. and Kidwell, W. R. (1973) Biochem. Biophys. Res. Commun. 53, 960–966.