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INVOLVEMENT OF POLYADP-RIBOSE POLYMERASE IN THE INITIATION OF PHYTOHEMAGGLUTININ INDUCED HUMAN LYMPHOCYTE PROLIFERATION

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SUMMARY : Nicotinamide (10 mM) or 3-aminobenzamide (5 mM) added at the onset of phytohemagglutinin (PHA) treated human lymphocyte cultures provoke a marked inhibition of the PHA induced DNA synthesis and cell proliferation as well as of poly(ADPR) polymerase activity. When the inhibitors of poly(ADPR) polymerase are added at a later stage of culture (48 h) no inhibition of the stimulation of DNA synthesis and cell proliferation by PHA in human lymphocyte cultures is observed. The intervention of ADP ribosylation at the initiation of DNA synthesis is suggested.

INTRODUCTION : Poly adenosine diphosphate ribose (polyADPR) polymerase is a chromosomal enzyme which uses the ADP-ribose moiety of NAD to synthesize poly(ADPR) covalently bound to nuclear proteins. The exact function fulfilled by the modification of nuclear proteins by mono- and poly(ADPR) remains unclear. It has been suggested that ADP-ribosylation is involved in the regulation of DNA synthesis, DNA repair, transcription and cellular differentiation (see for review 1-4).

It was previously shown that poly(ADPR) polymerase activity measured in permeabilized phytohemagglutinin (PHA) treated lymphocytes was stimulated in parallel to the enhancement of DNA synthesis and cell proliferation (5,6). In addition, we have shown (6) that 10 mM nicotinamide, a competitive inhibitor of polyADPR polymerase activity (7,8), added at the onset of the culture, provokes a marked decrease of the mitogen induced DNA synthesis and cell doubling together with the inhibition of poly(ADPR) polymerase activity. We suggested that inhibition of poly(ADPR) polymerase activity by a high concentration of

ABBREVIATIONS : ADPR : adenosine 5'-diphosphate ribose
NAD : nicotinamide adenine dinucleotide
poly(ADPR) : polymer of adenosine 5'-diphosphate ribose
PHA : phytohemagglutinin

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nicotinamide is involved in the blockage of the response of lymphocytes to PHA. In this paper we investigated at which period of PHA action, nicotinamide or 3-aminobenzamide blocks the response to PHA, in order to determine the stage at which ADP-ribosylation intervenes in the stimulation of DNA synthesis by PHA. Some of the data have been reported in a preliminary form (9,10).

MATERIALS : Lymphoprep was obtained from Nyegaard (Norway). Purified phytohemagglutinin (PHA) was purchased from Wellcome Research (England). Eagle's minimum essential medium and fetal calf serum were obtained from Gibco (USA). [^{3}H] Thymidine (80.1 Ci/mmol) and (adenine 2,8-^{3}H) NAD^{+} (2.96 Ci/mmol) were purchased from New England Nuclear Chemicals. (Carbonyl-^{14}C) nicotinamide (58 mCi/mmole) was from the Radiochemical Center Amersham (England). Rotiszint 22 scintillator was obtained from K. Roth (Germany), and GF/B glass fibre filters from Whatman (England). Unlabelled NAD as well as nicotinamide were obtained from Sigma Co (USA) and 3-aminobenzamide was from Kasei, Co. (Japan). All other chemicals were of analytical grade and were purchased either from Prolabo (France) or from Merck (Germany).

METHODS

Cell preparation : Human peripheral small lymphocytes were isolated from blood of normal donors. The cells were purified by density gradient centrifugation using lymphoprep and stimulated by PHA (1 µg/ml) according to the previously published procedure (6).

Thymidine incorporation : The [^{3}H] thymidine incorporation (1 µC/ml) in acid precipitable material was performed as described previously (6).

Nicotinamide incorporation : At appropriate time intervals after seeding, cells were pulsed for 30 minutes with [^{14}C] nicotinamide (0.2 µC/ml), the incorporation being linear till 60 min. The labelled cells were centrifuged, washed twice with PBS, and precipitated with ice-cold 0.6 N perchloric acid. The acid soluble material (0.5 ml) was mixed with 10 ml of Rotiszint 22 scintillator and counted in an Intertechnique Spectrometer SL 40.

Poly(ADP-ribose) polymerase assays : Enzymatic activity was measured in permeabilized cells (11) by incorporation of the ADP-ribose portion of [^{3}H] NAD^{+} into the acid insoluble material as previously described (12). One unit of enzyme activity was defined as 1 pmole (adenine-^{3}H) ADP-ribose incorporated into acid insoluble product per 5 min at 25°C. The specific activity is expressed as units per 10^{6} cells.

RESULTS : When 10 mM nicotinamide is added at the onset of the culture at the same time as PHA, a strong inhibition of both thymidine incorporation (Fig. 1B) and poly(ADPR) polymerase activity (Fig. 1A) is observed. In parallel the PHA induced increase in cell number is strongly inhibited (Table 1). These results are in agreement with those previously published (6).

In contrast, when nicotinamide is added to the lymphocytes culture medium 48 hours after the addition of PHA, no significant inhibition of the stimulation of DNA synthesis (Fig. 1B and Table 1) and of polyADPR polymerase activity

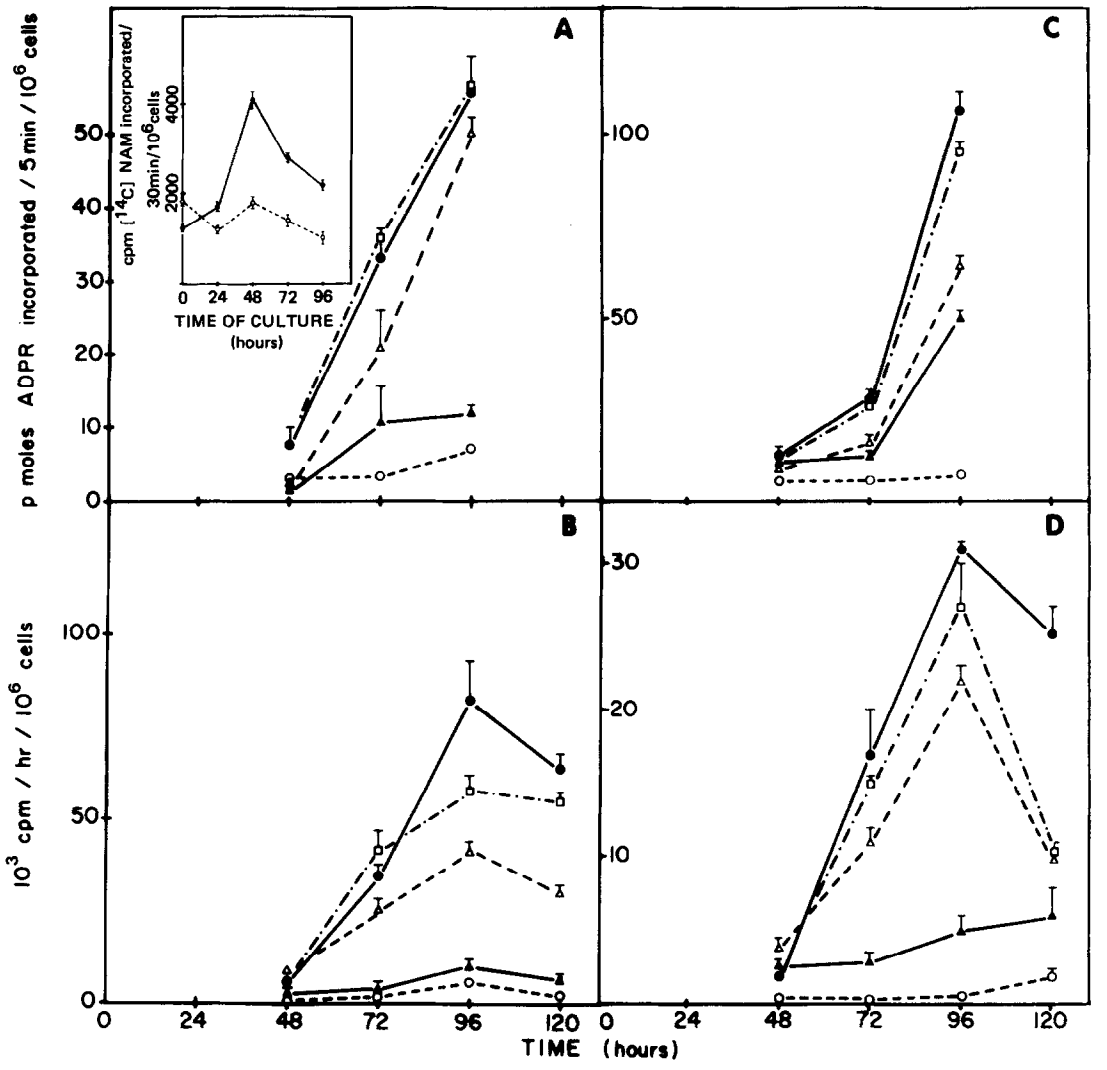

Figure 1 : Effect of 10 mM nicotinamide (A,B) or 5 mM 3-aminobenzamide (C,D) added at different times to PHA treated human lymphocyte cultures: (A,C) on polyADPR polymerase activity; (B,D) on thymidine incorporation rates. Insert : Nicotinamide uptake in human lymphocytes.
Control (o - - o), 1 µg/ml PHA treated lymphocytes in the absence (●—●) or in the presence of the inhibitor added at different times of culture : 0 h (▲—▲); 24h (△--△); 48 h (□··□). At appropriate time intervals cells were assayed for polyADPR polymerase activity and thymidine incorporation as described in the Methods section. Results are given as the mean ± S.E.

(Fig. 1A) by the mitogen is observed. When nicotinamide is added 24 hours after the addition of PHA, polyADPR polymerase activity as well as thymidine incorporation (Fig. 1) or cell proliferation (Table 1) reach an intermediary state between PHA stimulated lymphocytes and the same cells treated with nicotinamide at the seeding of the culture.

TABLE 1

	10^6 cells/ml at 120 h of culture	
	NAM treated cells	3AB treated cells
Control	0.28 ± 0.01	0.37 ± 0.07
PHA treated cells	1.11 ± 0.06	1.05 ± 0.24
PHA treated cells + inhibitor at 0 h	0.51 ± 0.15	0.57 ± 0.16
PHA treated cells + inhibitor at 24 h	0.76 ± 0.01	0.76 ± 0.09
PHA treated cells + inhibitor at 48 h	1.09 ± 0.24	0.89 ± 0.11

Effect of nicotinamide (NAM) or 3-aminobenzamide (3AB) on cell proliferation when added at different times to PHA treated human lymphocyte cultures. At 120 hours of culture viable cells, evaluated by the trypan blue exclusion test were counted. The initial cell number in all cases was 0.4 x 10^6 cells/ml for NAM treated cells and 0.5 x 10^6 cells/ml for 3AB treated cells. Results expressed as 10^6 cells/ml are the mean ± S.E.

Nicotinamide uptake is higher in PHA stimulated lymphocytes as compared to control cells with an optimum at the 48th hour of culture (insert Fig. 1). Therefore it seems unlikely that the lack of inhibition of PHA stimulation by nicotinamide added at the 48th hour of culture is due to a reduction of its uptake. The apparent lack of inhibition of the polyADPR polymerase activity measured, while the uptake of nicotinamide is increased can be explained by the fact that the determination of the enzymatic activity is performed *in vitro* (see Discussion section).

It was previously argued by Purnell and Whish (13) that in addition to the inhibition of polyADPR polymerase nicotinamide may act through a decrease of the nucleotide pool. These authors also suggested that 3-aminobenzamide can enter the cell and is a specific inhibitor which affects only poly(ADPR) polymerase activity. We investigated the effect of 3-aminobenzamide on PHA stimulation of thymidine incorporation, cell proliferation and on polyADPR polymerase activity in human lymphocytes.

5 mM 3-aminobenzamide, as for nicotinamide, produces a marked inhibition of PHA induced lymphocyte proliferation (Table 1) and thymidine incorporation (Fig. 1D) as well as poly(ADPR) polymerase activity (Fig. 1C) when added at the

onset of the culture together with PHA. Furthermore, 3-aminobenzamide added to cell cultures 48 hours after PHA does not inhibit the mitogen stimulation of poly(ADPR) polymerase activity (Fig. 1C) as well as of cell proliferation (Table 1), although thymidine incorporation is slightly inhibited (30%) after 96 hours of culture. When 3-aminobenzamide is added at the 24th hour of culture, the PHA stimulated thymidine incorporation (Fig. 1D) and cell proliferation (Table 1) as well as of poly(ADPR) polymerase activity (Fig. 1C) reach intermediary levels as compared to cells to which 3-aminobenzamide is added at the onset and 48th hour of culture.

DISCUSSION : Several reports suggest that ADP-ribosylation may be an important post-translational event involved in cell proliferation (see for review 14). In PHA-stimulated lymphocytes polyADPR polymerase activity has been reported to increase in parallel to mitogen stimulated DNA synthesis (6,15). We have previously shown (6) that nicotinamide, a competitive inhibitor of polyADPR polymerase inhibits DNA synthesis and the increase of cell number stimulated by PHA when added at the beginning of human lymphocyte cultures.

In this paper we investigated the stage at which ADP-ribosylation is involved in DNA replication of cultured human lymphocytes stimulated by PHA through a study of the effects of inhibitors of ADP-ribosylation : nicotinamide and 3 aminobenzamide added at different times (0h, 24h and 48h) to the culture medium. The variations in the rate of DNA synthesis followed by the determination of thymidine incorporation paralleled the changes observed in cell number.

The results presented in this paper demonstrate that ADP-ribosylation is a necessary step for the initiation of DNA replication induced by PHA. This conclusion is based on the following findings : when the inhibitors of poly ADPR polymerase are added at the same time as PHA, thymidine incorporation and cell proliferation are inhibited, whereas when inhibitors are added 48hrs after addition of PHA no inhibition of the above parameters is observed indicating that these cells have reached a specific stage of the cell cycle where ADP-ribosylation is no more an absolute requirement.

Nicotinamide or 3-aminobenzamide added to the culture medium 24h after PHA reduces only partly the thymidine incorporation and cell number increase. This may be due to the fact that lymphocytes are not synchronized cells and that a population of cells is at a stage at which ADP-ribosylation is no more necessary in the replication process.

The lack of inhibition of polyADPR polymerase activity when the inhibitors are added 48 hours after PHA (Fig. 1A and C), when nicotinamide uptake is the highest, may seem surprising. It is likely that the polyADPR polymerase activity measured in permeabilized cells under our experimental conditions does not reflect the *in vivo* state but rather the amount of enzyme molecules present in the assay mixture. The observed inhibition of polyADPR polymerase activity in cells to which the inhibitors were added at the same time as PHA can be explained by the fact that in these cells the response to PHA as well as the activation of polyADPR polymerase molecules have been blocked.

Recently, Johnstone and Williams (16) presented data concerning the intervention of ADP-ribosylation in the early stage of mitogen activation in human lymphocytes. These authors relate their finding to rejoining of single strand breaks in lymphocyte DNA, in correlation to a specific rearrangement of genetic material. The same authors conclude that ADP ribosylation does not intervene in cell proliferation since the inhibitors of polyADPR polymerase do not affect the proliferation of undifferentiated lymphocytes. It is well known that the vast majority of lymphocytes obtained either from the blood or from lymphoid organs do not divide *in vitro* satisfying the definition of G_0 cells. Upon exposure to lectin in culture these cells are able to enter the cell cycle (17). Based on our results concerning thymidine incorporation and cell number our data suggest that ADP ribosylation is involved in an early stage of DNA synthesis and consequently of cell proliferation induced by PHA.

Previously, we have shown (18) that ADP-ribosylation produces a relaxation of the native chromatine structure *in vitro*. It is conceivable that relaxed chromatine renders the DNA more accessible to the enzymes intervening in the replication process. Moreover, we have found (19) that the activity

of topoisomerase I, one of the enzymes which controls the conformation of DNA and which is considered to be also involved in DNA replication (20-21), is inhibited following its ADP ribosylation *in vitro*. The identification of ADP - ribosylated proteins during the induction of DNA synthesis in PHA stimulated lymphocytes will undoubtly be of great help for our understanding of the role of ADP-ribosylation in this process.

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REFERENCES

1. Hilz, H., and Stone, P. (1976) Rev. Physiol. Biochem. Pharmacol. 76, 1-58.
2. Hayaishi, O., and Ueda, K. (1977) Ann. Rev. Biochem. 46,95-116.
3. Purnell, M.R., Stone, P.R., and Whish, W.J.D. (1980) Biochem. Soc. Trans. 8, 215-227.
4. Mandel, P., Okazaki, H., and Niedergang, C. (1982) Prog. Nucl. Acid. Res. and Mol. Biol. 27, pp. 1-51, Academic Press, New York.
5. Berger, N.A., Adams, J.W., Sikorski, G.W., Petzold, S.J., and Shearer, W. T. (1978) J. Clin. Invest. 62, 111-118.
6. Rochette-Egly, C., Ittel, M.E., Bilen, J., and Mandel, P. (1980) FEBS Lett. 120, 7-11.
7. Preiss, J., Schlaeger, R., and Hilz, H. (1971) FEBS Lett. 19, 244-246.
8. Niedergang, C., Okazaki, H., and Mandel, P. (1979) Eur. J. Biochem. 102, 43-57.
9. Ittel, M.E., Jongstra-Bilen, J., Rochette-Egly, C., and Mandel, P. Eur. Meeting on ADP-Ribosylation of Proteins, Brighton (G.B.) Dec. 1981, abstr. n° 17.
10. Ittel, M.E., Jongstra-Bilen, J., Rochette-Egly, C., and Mandel, P. First Eur. Congress on Cell Biology, Paris, July 1982, abstr. n° 209.
11. Berger, N.A., Weber, G., and Kaichi, A.S. (1978) Biochim. Biophys. Acta 519, 87-104.
12. Bilen, J., Ittel, M.E., Niedergang, C., Okazaki, H., and Mandel, P. (1981) Neurochem. Res. 6, 1253-1263.
13. Purnell, M.R.,and Whish, M.J.D. (1980) Biochem. J. 185, 775-777.
14. Koide, S.S. (1982) ADP-Ribosylation Reaction, pp. 361-371, Academic Press, New York.
15. Berger, N.A., Adams, J.W., Sikorsky, G.W., and Petzold, S.J. (1978) J. Clin. Invest. 62, 111-118.
16. Johnstone, A.P., and Williams, G.T. (1982) Nature 300, 368-370.
17. Richman, D.P. (1980) J. Cell Biol. 85, 459-465.
18. Poirier, G.G., de Murcia, G., Jongstra-Bilen, J., Niedergang, C., and Mandel, P. (1982) Proc. Natl. Acad. Sci. USA 79, 3423-3427.
19. Jongstra-Bilen, J., Ittel, M.E., Niedergang, C., Vosberg, H.P., and Mandel, P. Eur. J. Biochem. (in press).
20. Gellert, M. (1981) Ann Rev. Biochem. 50, 879-910.
21. Duguet, M. (1981) Biochimie 63, 649-669.