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REGULATION OF REPAIR OF NATURALLY OCCURRING DNA STRAND BREAKS IN LYMPHOCYTES

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Mouse lymphocytes have been shown to contain DNA strand breaks that were repaired within 2h of onset of culture with mitogen. Inhibitors of ADP ribosylation prevented this repair and blocked cell proliferation. The mitogen concanavalin A caused the internal concentration of NAD^+, the substrate of the ADP ribose polymerase, to rise to about double that of resting cells within 45 min of stimulation. Addition of 300 μm nicotinamide to the culture in absence of mitogen also resulted in a similar increase in internal [NAD^+], resulting in increased ADP ribosylation activity (measured in permeabilized cells) and in joining of DNA strand breaks; however, none of the subsequent events of lymphocyte activation such as blast transformation and DNA synthesis occurred. These findings indicate that (1) cellular [NAD^+] is a rate limiting factor in repair of DNA strand breaks in resting lymphocytes and (2) this repair is necessary but not sufficient for lymphocyte proliferation.

Recent reports (1,2,3) have proposed that change in the number of DNA strand breaks, regulated by ADP ribose polymerase, may be a mechanism for altering gene expression as cells differentiate; indeed this was anticipated several years before (4). Rapidly dividing chick myoblasts were found to acquire DNA breaks as they differentiated to become multinucleated muscle fibers (1). Human peripheral blood lymphocytes were reported to contain DNA breaks which were repaired within 8h of mitogenic stimulation (2). The nucleoid sedimentation method used in this work did not identify the number of breaks present in resting cells; the slower observed rate of nucleoid sedimentation in neutral gradients following stimulation with mitogen was attributed to the increased supercoiling of DNA caused by a drop in number of breaks. Neutral gradients permitted the distinction between strand breaks and alkali labile bonds.

Using a more accurate technique for the estimation of DNA strand breaks, we have shown that about 3000 strand breaks per cell, in resting mouse splenocytes, were repaired

Abbreviations : NAD , nicotinamide adenine dinucleotide; Con A, concanavalin A ; LPS , lipopolysaccharide; TdR , thymidine

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within 2h of onset of culture with mitogen (5). Additional data (6) suggested that in both resting and proliferating lymphocytes there was a continuous production and repair of DNA strand breaks, and the steady state number of these breaks decreased after mitogenic activation. Evidence was presented in the case of both human and mouse lymphocytes that ADP ribosylation, implicated in efficient excision repair (7), was involved in the repair of strand breaks during mitogen induced proliferation (2,5).

The benzamide inhibitors of ADP ribose polymerase (8) prevented the mitogen-induced repair of strand breaks in human and in mouse lymphocytes, as well as their subsequent proliferation (2,5). This, together with the recent demonstration that resting lymphocytes contained low levels of [NAD^+] which increased following mitogen treatment (9), suggested to us that cellular levels of NAD^+, a substrate for ADP ribose polymerase (10), may be rate limiting for ADP ribosylation and therefore for repair of strand breaks in resting lymphocytes. Our data support this hypothesis.

Materials and Methods

Cell Preparation and Culture

Balb/c male mice, 8-12 weeks old, were killed by cervical dislocation and their spleens disrupted through a wire mesh screen. Red blood cells were removed by lysis with 0.83% ammonium chloride. Cells were cultured in RPMI 1640 medium with 10% fetal calf serum, and 2 mM glutamine, at a density of 2 x 10^6 cells/ml.

Detection of DNA Strand Breaks

DNA strand breaks were detected using the fluorometric analysis of DNA unwinding technique developed by Birnboim and Jevcak (11). After denaturation at pH 12.8 for 1 h, the cell lysate was adjusted to pH 11, and ethidium bromide was added. Under these conditions, ethidium bromide binds to, and fluoresces selectively in, double stranded DNA. From the percentage of double stranded DNA remaining after alkaline treatment, one can estimate the number of DNA strand breaks by using a calibration curve obtained from cells treated with various doses of gamma radiation [500 rads of γ radiation produce 6000 single strand breaks per diploid genome (12)].

Assays for ADP Ribosylation, and NAD^+ Levels

ADP ribosylation was measured as incorporation of [^{3}H] NAD^+ into the acid-insoluble fraction of permeabilized cells according to the method of Berger and Johnson (13).

NAD^+ levels were measured according to the method of Nisselbaum and Green (14). This method distinguishes oxidized and reduced forms of NAD.

Results

In agreement with our previous report (5), Table 1 shows that a mixed population of mouse splenocytes contained DNA strand breaks which were repaired within 2h after

TABLE 1

Repair of strand breaks in lymphocytes from normal spleen, thymus, and "nude" spleen after 2h treatment with mitogen

Cell Source	Treatment	%Double Stranded DNA[1]	% Blasts at 48h[2]	[^{3}H] Tdr incorp./10^6 cells at 48h cpm
Normal Spleen	Resting	46 ± 3	5	2,400 ± 900
	Con A 2 μg/ml	71 ± 5	82	42,300 ± 6,000
	LPS 20 μg/ml	66 ± 4	46	4,800 ± 1,200
Thymus	Resting	62 ± 4	3	1,200 ± 150
	Con A 3 μg/ml + IL-2[3] 8 units/ml	65 ± 4	65	28,400 ± 900
Nude Spleen	Resting	48 ± 4	14	1,700 ± 600
	Con A 2 μg/ml	55 ± 2	17	2,400 ± 400
	LPS 20 μg/ml	56 ± 3	60	11,400 ± 2,000

[1]This refers to the % double stranded DNA remaining after 1h denaturation in alkali; values are inversely proportional to number of strand breaks. Using a calibration curve obtained from cells treated with various doses of γ radiation, the difference in % double stranded DNA observed in spleen compared to thymus was found to represent a difference of 3000 to 4000 strand breaks per diploid genome.

[2]Blasts were defined as any cell with diameter larger than 10 μm.

[3]IL2 = Interleukin-2.

mitogenic stimulation. This repair of breaks was induced by either T or B cell mitogens (Con A and LPS respectively). The predominantly B cells from spleen of athymic nude mice contained DNA breaks that were repaired after stimulation with LPS. Repair also occurred after treatment of these B cells with the T cell mitogen Con A, yet no incorporation of ^{3}H-TdR occurred at 48h. The extent of repair in B cells after incubation with Con A varied from 30% to 100% of that observed with LPS. Table 1 also compares the effect of mitogen on splenic and thymic lymphocytes. Unstimulated cells from the thymus contained fewer breaks per genome than those from the spleen of normal or nude mice. After treatment with Con A and interleukin 2, thymocytes did not undergo further decrease in strand breaks, but they did proliferate.

In order to test the hypothesis that NAD^+, the substrate for ADP ribose polymerase, is limiting for ADP ribosylation (and thus for repair) in resting splenic lymphocytes, the intracellular concentration of NAD^+ was varied experimentally by adding its precursor nicotinamide to the external growth medium. Figure 1 (inset) shows that 2h incubation with 330 to 420 μM nicotinamide resulted in a 2-fold increase in the overall intracellular

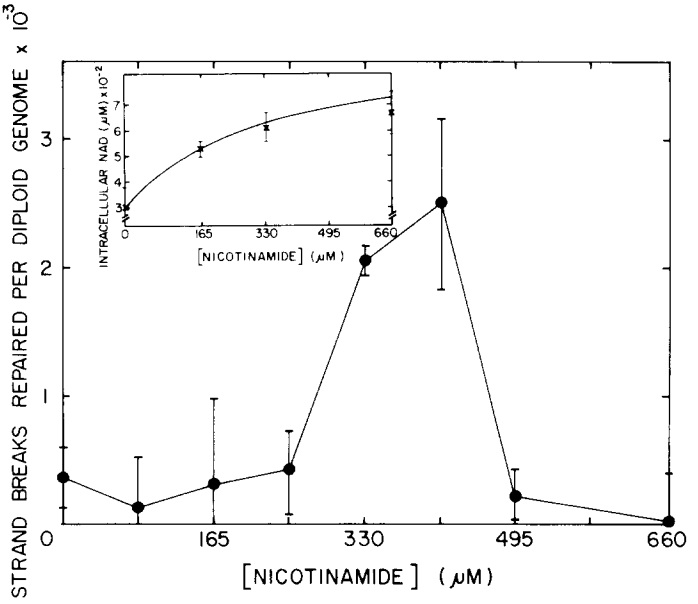

Figure 1. Strand breaks were assayed after 2h incubation with various concentrations of nicotinamide in the external growth medium. Intracellular levels of NAD^+ were measured in the same samples and are shown in the inset. There was no change in cell viability in the presence of nicotinamide.

concentration of NAD^+. Figure 1 shows that under these conditions strand breaks were repaired in the absence of Con A. However, the incorporation of thymidine into DNA noted in the stimulated control cells at 48h was either absent or reached only 10% of control levels. Repair was not induced with higher concentrations of nicotinamide, probably due to its well known effect as a competitive inhibitor of ADP ribose polymerase (15). Nicotinamide-induced repair did not occur if the ADP ribose polymerase inhibitor, methoxybenzamide, was also present.

The hypothesis that NAD^+ may be limiting for repair in resting cells was supported by the finding that after incubation with Con A the intracellular levels of NAD^+ increased by about 2-fold (Figure 2), a phenomenon also noted in the case of peripheral blood lymphocytes (9). There was no increase in nicotinamide uptake noted after Con A stimulation, which indicates that the rise in intracellular NAD^+ was not the result of increased transport. Figure 3 shows that the 2-fold increase in intracellular levels of NAD^+ (compare Fig. 2) was found to coincide closely with the increase in ADP ribosylation capacity measured in permeabilized cells. Both of these events precede the major burst of repair induced by Con A.

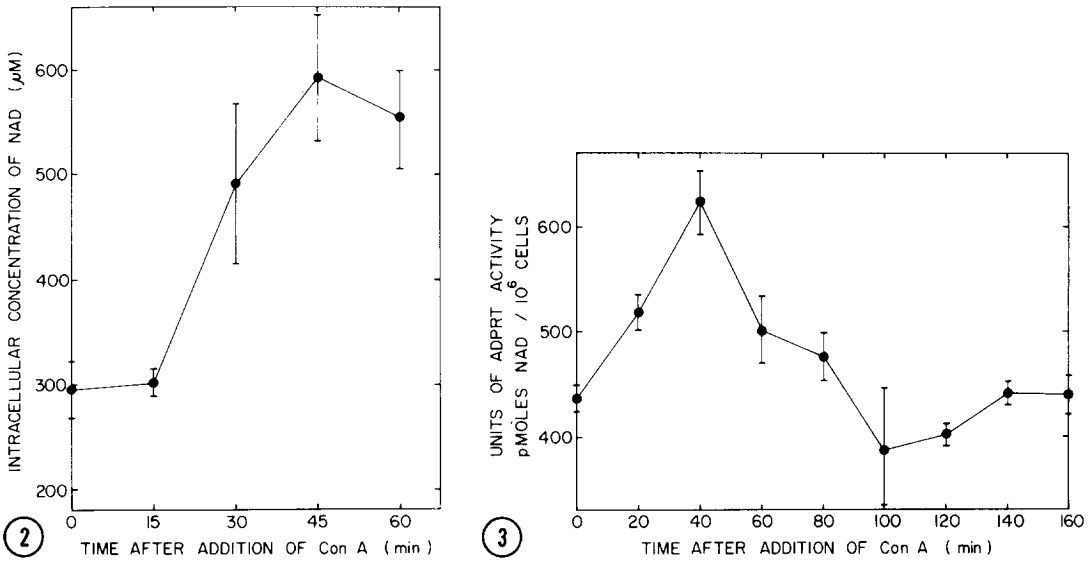

Figure 2. Con A was added to cells at staggered times from 0 to 60 min, and the intracellular concentration of NAD^+ was assayed in all samples at 60 min.

Figure 3. Con A was added to cells at staggered times from 0 to 160 min, and ADP ribosylation was assayed in all samples at 160 min, as incorporation of $[^3H]$ NAD into the acid soluble fraction of permeabilized cells.

Discussion

It has been hypothesized that an increase in the number of DNA strand breaks is an important part of cell differentiation (3). Our data show that resting splenocytes contain more breaks than thymocytes, an observation consistent with the above hypothesis, since the majority of thymocytes appear to be immature, and not yet fully differentiated (16).

Following addition of Con A to mouse splenocytes, there is a good correlation in time of the increase in cell $[NAD^+]$ with the increase in ADP ribosylation activity and with the decrease in number of DNA strand breaks. Since this decrease also occurs if NAD^+ levels are raised by exogenously supplied nicotinamide in the absence of Con A, we conclude that $[NAD^+]$ is a rate limiting factor for mitogen-induced repair in lymphocytes. The fact that nicotinamide-induced repair did not occur in the presence of methoxybenzamide supports the conclusion that repair involves ADP ribosylation. This accords with the finding of Kol and Ben-Hur (17) that nicotinamide in the external growth medium protected human lymphocytes against γ radiation damage; these authors suggested that NAD^+ may be rate limiting for excision repair in these cells. Berger and Sikorski (18) reported that nicotinamide stimulated DNA repair synthesis in human lymphocytes treated

with UV radiation. Other possible factors limiting repair in resting lymphocytes are the activity of the ADP ribose polymerase and/or ligase and β polymerase, and location of the breaks in the DNA structure.

Although increased cellular levels of NAD^+ induced DNA repair in resting lymphocytes, the latter did not proceed into further stages of activation. Further, the T cell mitogen Con A induced repair in the predominantly B cell population from the spleen of athymic nude mice, but the cells did not form blasts or proliferate (Table 1). We have shown elsewhere (6) that there is a continual production and repair of DNA strand breaks in resting and stimulated mouse splenic lymphocytes, with a reduction in the steady state level of breaks after stimulation with mitogen. We conclude that this is at least partly due to the increase in cell $[NAD^+]$ that follows stimulation and that this causes repair of DNA strand breaks that is necessary but not sufficient for lymphocyte activation and proliferation.

Acknowledgements

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