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# *In vivo* exposure of Swiss albino mice to chronic low dose of dimethylnitrosamine (DMN) lowers poly-ADP-ribosylation (PAR) of bone marrow cell and blood lymphocyte proteins

L. Kma and R. N. Sharan

*Radiation and Molecular Biology Unit, Department of Biochemistry, North-Eastern Hill University, Umshing, Shillong 793 022, India*

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## Abstract

Efforts to identify an easy and convenient biomarker of carcinogenesis with potentials of application in mass screening program continue. In a series of investigations on mice exposed to different carcinogens, poly-ADP-ribosylation (PAR) of cellular proteins of different tissues has been shown to be a potential biomarker of carcinogenesis. Because blood based biomarker of carcinogenesis offers significant advantage in its use in a cancer screening program, this investigation was undertaken to find correlations between initiation of carcinogenesis and PAR of bone marrow cell (BMC) and blood lymphocyte (BL) proteins in mice chronically exposed to low dose of dimethylnitrosamine (DMN) for up to four weeks *in vivo*. The exposure was either alone or in combination with 3-aminobenzamide (3-AB), an inhibitor of PAR. Total PAR of cellular proteins and of histone H1 protein were monitored by slot and Western blot immunoprobe assays, respectively. The PAR of total cellular proteins as well as of histone H1 was down-regulated in duration of exposure dependent manners. The results suggest that BMC and BL mirrored status of PAR in other tissues. This finding opens up the possibility of using PAR as a biomarker of carcinogenesis in a blood based test utilizing immunoprobe assay of cellular PAR. (Mol Cell Biochem **288:** 143–149, 2006)

*Key words*: poly-ADP-ribosylation (PAR), dimethylnitrosamine (DMN), mice, slot and western blot, immunoprobe assay of PAR, *in vivo*

*Abbreviation*: 3-AB, 3-aminobenzamide; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium; BL, blood lymphocytes; BMC, bone marrow cells; DMN, dimethylnitrosamine; H1, histone H1; $NAD^+$, nicotinamide adenine dinucleotide; PAb, polyclonal antibodies; PAR, poly-ADP-ribosylation; PARP, poly-ADP-ribose polymerase; PVDF, poly-vinyl dinitrofluoride; WH, whole homogenate

## Introduction

Poly ADP-ribosylation (PAR) is an enzyme catalyzed and reversible post-translational modification of mainly chromosomal proteins [1–3]. PAR reaction involves successive addition of ADP-ribose moieties donated by endogenous nicotinamide adenine dinucleotide ($NAD^+$) to target proteins or its removal [1]. The resulting heterogeneous ADP-ribose polymer on the modified protein has been shown to affect the structural and functional activities of chromatin. Therefore,

*Address for offprints*: R. N. Sharan, Radiation and Molecular Biology Unit, Department of Biochemistry, North-Eastern Hill University, Umshing, Shillong 793 022, India (E-mail: rnsharan@nehu.ac.in)

PAR has been shown to be intricately involved in carcinogenesis [2–9].

In a series of studies directed to expose the link between PAR and carcinogenesis, we have used a simple yet sensitive slot and Western blot immunoprobe assay of PAR using polyclonal antibody (PAb) against heterogeneous ADP-ribose polymers to measure it in relation to carcinogenesis [10, 11]. The PAR of total cellular proteins as well as of histone proteins of liver and spleen cells were significantly lowered during initiation of carcinogenesis in mice exposed to dimethylnitrosamine (DMN) [4, 7, 8, 10]. Similarly, aqueous extract of betel nut or arecoline was also found to lower PAR of different proteins in mice [4–6, 12]. In Dalton's lymphoma ascites tumorigenesis, the PAR of histone proteins was progressively lowered during later stage of cancer development [9]. Based on these observations, it has been hypothesized that PAR would be imminently suitable biomarker of carcinogenesis and could be potentially used for mass screening of cancer in a population [7–11]. However, use of tissue samples or biopsies in a cancer screening would make the process difficult to apply in mass screening program due to required surgical intervention. A blood based assay of PAR would naturally be most non-invasive and, thus, easy to perform making it a highly desirable proposition. Our preliminary report on level of PAR of mouse blood lymphocyte (BL) protein has confirmed the trend [7]. This report presents results of a detailed study wherein PAR of cellular proteins of bone marrow cells (BMC) and BL were monitored during the initiation phase of carcinogenesis in mice chronically exposed to DMN *in vivo*. To get deeper insight, another group of mice on chronic DMN exposure was simultaneously administered with 3-aminobenzamide (3-AB), a potent inhibitor of PAR reaction [13, 14].

## Methods and materials

*Chemicals*

All chemicals were of analytical grade and were used without further purification. All required solutions were prepared in double-distilled water. DMN and 3-AB were purchased from Sigma Chemical Co., USA and Biocoll (1.090 g $ml^{-1}$) from Biochrome KG, Germany.

*Animals*

Young (6–8 weeks old) Swiss albino mice (Balb/C), used in the investigation, were maintained on standard mouse pellet (Pranav Agro Products, Delhi) and drinking water *ad libitum* in a temperature (25 °C) and day-light (12 h cycle) controlled animal room.

*Dose and protocol of dimethylnitrosamine (DMN) and 3-aminobenzamide (3-AB) administration*

The administration of DMN followed a protocol already reported [7, 10]. Briefly, mice were exposed to DMN at a dose rate of 10 mg $kg^{-1}$ body weight in drinking water *ad libitum* in a chronic exposure protocol. To another group of mice being chronically exposed to DMN, 3-AB was simultaneously administered at a dose rate of 5.45 mg $kg^{-1}$ body weight by way of weekly intraperitoneal (ip) injection. Mice were sacrificed for analysis by cervical dislocation at 0 (control), 1, 2, 3 and 4 weeks after initiation of the exposure to cover initiation phase of carcinogenesis [15]. Age and sex matched unexposed mice served as controls.

*Preparation of whole homogenates (WH) of blood lymphocytes (BL) and bone marrow cells (BMC)*

Immediately after sacrifice, blood was collected from mouse heart using heparinized syringe and collection tube. Simultaneously, the femurs (thigh) bones were also excised out. For preparing BL, equal volume of the collected blood and RPMI-1640 were mixed. The mixture was gently layered over Biocoll separation medium in a centrifuge tube. It was centrifuged at 400 × g for 40 min at 4 °C [16, 17]. The clear lymphocyte layer at the interphase was collected using a syringe and washed with equal volume of PBS by spinning the suspension at 250 × g for 10 min at 4 °C. The pellet was homogeneously suspended in 1 ml of PBS. For preparing BMC, the femurs were cleared of the attached soft tissues and the heads of femur shaft were chopped off. With 2 ml PBS in a syringe, the marrow was thoroughly flushed out in a clean test tube and made into single cell suspensions. The number of cells in the suspensions were counted using Burker's chamber as described [9].

The method of Rosenberg [18], with some modification, was used to prepare whole homogenates (WH) of BMC and BL for further investigations. Briefly, the WH was prepared using motorized tissue homogenizer [7]. All BMC recovered from 12 femurs or $6 \times 10^6$ BL were used to prepare WH. Protein quantification in the WH was done by Bradford method [19] with BSA as a standard.

*Slot and Western blot immunoprobe assay of poly-ADP-ribosylation (PAR) of BMC and BL protein*

The details of the method have been published recently [10, 11]. Briefly, WH of BMC and BL were either slot blotted on poly-vinyl dinitrofluoride (PVDF) or subjected to 12% SDS-PAGE followed by Western blotting on PVDF. The blotted PVDF were immunoprobed with PAb against ADP-ribose

polymer as described [10]. The blotted PVDF was stained using BCIP/NBT system. The band intensities of poly-ADP-ribosylated proteins were quantified in terms of net pixel intensity and analyzed using Bio-Rad imaging densitometer and molecular analyst 1-D software. While the immunoprobed slot blot gave the measure of PAR of total WH proteins, the immunoprobed Western blot showed PAR of individual cellular proteins. Replica PVDF were also stained with India ink to visualize total slotted or Western blotted proteins. Concentration corrected net intensities of PAR from immunoprobed PVDF were used to calculate differences as % of the controls. Data are shown as mean ± SEM.

## Results

Each treatment point in an experimental group consisted of 6 age-matched female mice. The whole set of experiment was done together. The slot and Western blot immunoprobings were done in triplicate and the entire experiment was independently repeated at least one more time. Until the end of the observation period, no mouse exhibited illness of any kind. For each set of experiment, WH containing equal amounts of protein was used. Usually 2–4 $\mu$g protein in 100 $\mu$l volume was used for slot blotting. For Western blotting, 35–40 $\mu$g protein in about 25 $\mu$l was the starting concentration loaded on SDS-PAGE. The blotted PVDF were immunoprobed to visualize poly-ADP-ribosylated proteins. Simultaneously, their replica PVDF were stained with India ink to visualize total blotted proteins.

*Effect of DMN exposure on PAR of histone proteins*

Figure 1 shows the immunoprobed and India ink stained Western blotted PVDF for WH of BMC (A) and BL (B). The India ink stained PVDF (right panels) visually show consistency in the amounts of protein (sample) blotted. In contrast, their immunoprobings (left panels) reveal a DMN exposure period dependent lowering of PAR. This trend was much less pronounced in BMC (Fig. 1A) as compared to BL (Fig. 1B). In both cases, however, the only histone protein to be prominently poly-ADP-ribosylated was H1. PAR of other histone proteins were generally very low or not detectable.

*Effect of DMN+3-AB exposure on PAR of histone proteins*

Figure 2 shows the immunoprobed and India ink stained Western blotted PVDF for WH of BMC (A) and BL (B).

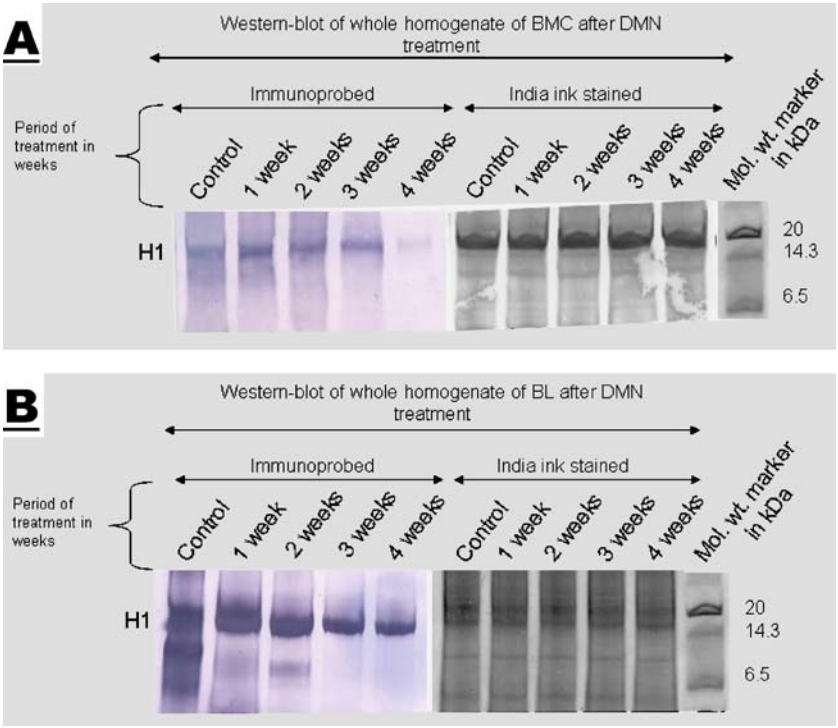


*Fig. 1.* Western blots of whole homogenate of bone marrow cells (BMC; **A**) and blood lymphocytes (BL; **B**) of control and DMN exposed mice. One of the electroblotted PVDF membranes was immunoprobed for PAR of individual resolved proteins (left blot) while its replicum was stained with India ink for total proteins (right blot). A protein molecular weight marker (extreme right lane) was used to identify poly-ADP-ribosylated histone H1 protein.

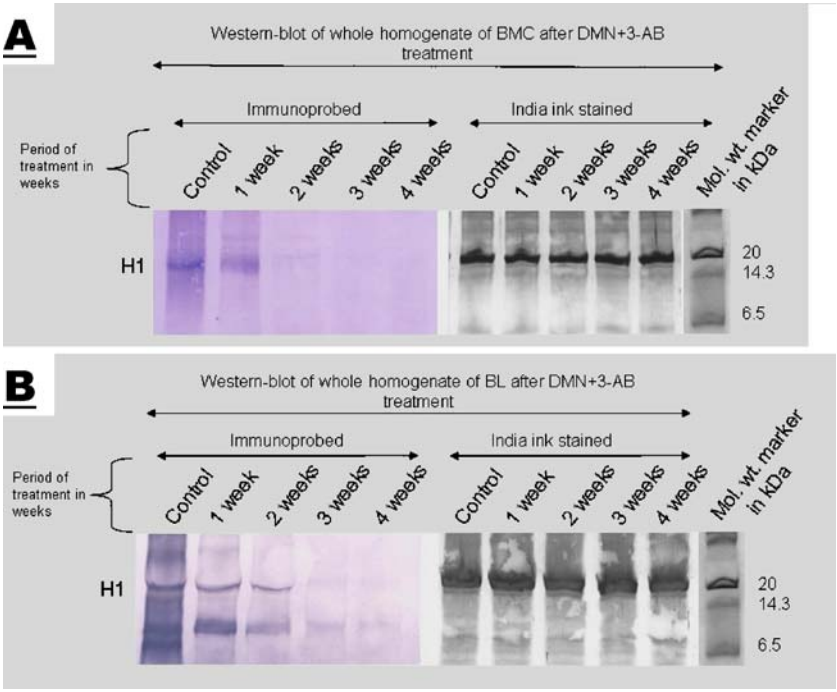


*Fig. 2*. Western blots of whole homogenate of bone marrow cells (BMC; **A**) and blood lymphocytes (BL; **B**) of control and DMN+3-AB exposed mice. One of the electroblotted PVDF membranes was immunoprobed for PAR of individual resolved proteins (left blot) while its replicum was stained with India ink for total proteins (right blot). A protein molecular weight marker (extreme right lane) was used to identify poly-ADP-ribosylated histone H1 protein.

Again, the India ink stained PVDF (right panels) visually show consistency in the amounts of protein (sample) blotted. Upon immunoprobing (left panels), a relatively low level of PAR of histone H1 was detected in both BMC (Fig. 2A) and BL (Fig. 2B). In this case also, DMN+3-AB exposure period dependent lowering of PAR of histone H1 was apparent.

*Quantitative analysis of effects of DMN and DMN+3-AB exposures on PAR of histone H1*

The plots in Fig. 3 show the results of quantitative analysis of PAR of histone H1 from the Western blots for BMC (A) and BL (B). Both exhibited progressive exposure period dependent drops in the levels of PAR of histone H1 as compared to the controls. At week 4, the drop was between 75 and 80% in BMC (Fig. 3A) and in BL (Fig. 3B). Simultaneous presence of 3-AB along with DMN (DMN+3-AB group) only aggravated the lowering, more prominently (>80%) in BMC (Fig. 3A; open squares) than in BL (Fig. 3B; open triangle).

*Effect of DMN and DMN+3-AB exposure on total PAR of all cellular proteins*

Figure 4 shows the India ink stained (I, II, III, IV) and immunoprobed (IA, IIA, IIIA, IVA) slot blotted PVDF for WH of BMC and BL of mice exposed to DMN alone or to DMN+3-AB. While the respective India ink stained slot blotted PVDF visually show consistency of total protein slotted within respective treatment groups, their replica, immunoprobed PVDF show progressive lowering of total PAR of cellular proteins slotted. The quantification plot of total poly-ADP-ribosylated cellular proteins from these slots makes the trend very clear (Fig. 5). The reduction was, on expected lines, more pronounced in BMC as well as BL of DMN+3-AB exposed mice reaching 70 to >80% of the controls in the 4th week, respectively, as compared to DMN treated group where it was approximately between 45 and 65%.

## Discussion

The involvement of PAR of cellular proteins in carcinogenesis as well as in other related molecular events including cell cycle has been shown [2, 3, 5, 12, 20]. The involvement of PAR with carcinogenesis is further supported by many factors. For instance, it has been shown that diethylnitrosamine and betel nut, two carcinogens specific to different target tissues, initiated similar initial molecular events on HMG proteins [4]. Since the hallmark of carcinogenesis is known to be uncontrolled cell proliferation and dedifferentiation [21], PAR of cellular proteins, especially chromosomal proteins, was

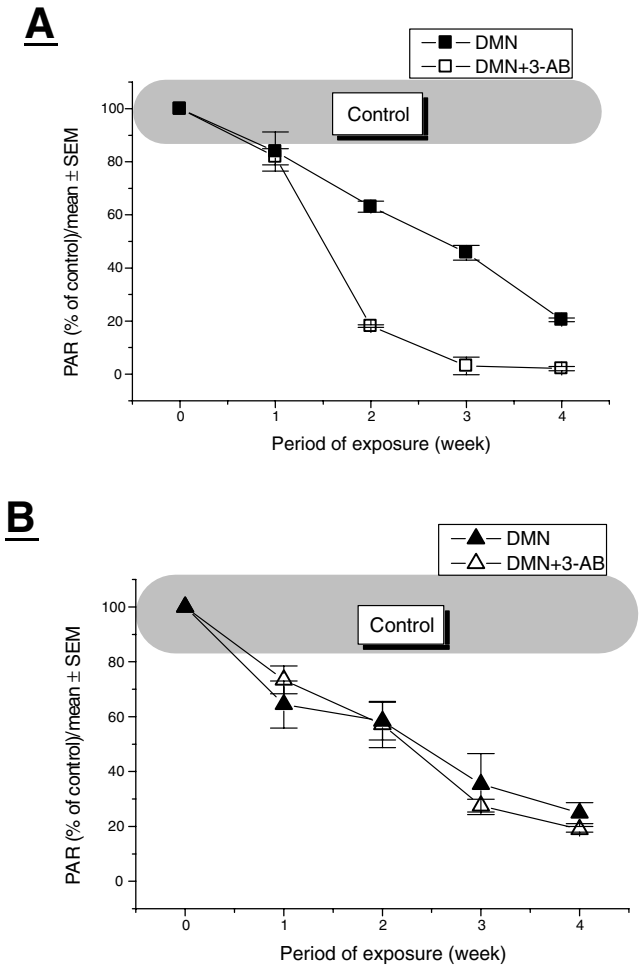


*Fig. 3.* Quantification plot of level of PAR of histone H1 protein of bone marrow cells (BMC; **A**) and blood lymphocytes (BL; **B**) as a function of period of exposure to DMN alone (closed symbols) or DMN+3-AB (open symbols). The graph of mean intensity (pixel density) has been plotted as % of control after densitometric quantification of the immunodetected Western blots (Figs. 1 and 2). The level of PAR in age and sex matched control mice is shown as a shaded bar.

also shown to be a key regulator of structural and functional status of chromatin [1, 2, 5, 6]. In order to support cell proliferation, the chromatin organization must undergo structural and functional alterations during initiation of carcinogenesis [20, 21]. Thus, PAR of chromosomal proteins is very likely to be influenced during carcinogenesis. This hypothesis was the basis of our continuing investigation and we do find statistically significant correlation between PAR and carcinogenesis [7–10]. To measure the metabolic level of PAR of cellular proteins, we have developed sensitive, specific and convenient slot– and Western blot immunoprobe assays using PAb against native, heterogeneous ADP-ribose polymer [10, 11]. The sensitivity of the PAb was found to be higher than monoclonal Ab against ADP-ribose for assay of cellular PAR [11]. Therefore, the assay used in this investigation is imminently suitable for assay of metabolic level of cellular PAR. DMN has been reported to down-regulate total cellular PAR as well as PAR of histone proteins of liver and spleen cells during initiation of carcinogenesis [7, 8, 10]. Even during late stages of carcinogenesis, as in Dalton's lymphoma induced ascites tumorigenesis, PAR of cellular proteins correlated negatively with carcinogenesis [9]. Based on these observations, we have postulated that PAb based immunoprobe assay of PAR of cellular proteins could be a useful biomarker, which could potentially be used in mass cancer detection and screening programs [7–11]. However, obtaining tissue or biopsy samples for the assay of PAR in a mass screening program would involve massive surgical interventions making the whole approach impractical. A blood based assay of PAR would involve minimum surgical intervention and would be practical for mass screening program. Since our preliminary results showed that PAR of BL proteins mirrored the trend shown by a transformed tissue [7], this detailed investigation was undertaken to find the correlation between PAR and BL proteins during initiation stage of carcinogenesis induced by DMN in mice *in vivo*. As the progenitor of BL is BMC, PAR of BMC proteins was also monitored in the investigation. The observations were made for up to 4 weeks following DMN exposure as it has been shown earlier that initiation phase of carcinogenesis is completed in this period [15].

Unlike our earlier observation of preferential, high degree PAR of histone group of proteins in liver and spleen cells during initiation phase of DMN induced carcinogenesis in mice [8–10], both BMC (Fig. 1A) and BL (Fig. 1B) showed significantly less PAR of histone group of proteins. Histone H1 seemed to be the only protein significantly poly-ADP-ribosylated in these two tissues during initiation phase of DMN induced carcinogenesis in mice. The level of PAR of histone H1, however, progressively went down with progression of DMN exposure period when equal amount of WH samples of BMC and BL were immunoprobed (Fig. 1; compare India ink stained blots with the immunoprobed blots). The plot of PAR of histone H1 against DMN exposure period shows significant lowering with progression of initiation of carcinogenesis (Fig. 3).

3-AB is a physiological inhibitor of anabolic enzyme of PAR reaction, poly-ADP-ribose polymerase (PARP) [13, 14]. Therefore, in its presence, the cellular PAR is inhibited. Consequently, when 3-AB was simultaneously administered with DMN, the level of PAR of BMC and BL proteins were, as expected, lower than DMN alone (compare Western blots in Figs. 1 with 2). However, the quantitative lowering of PAR of BMC and BL proteins induced by DMN+3-AB exhibited similar trend as DMN alone. This is evident by the quantification plot of PAR of histone H1 (Fig. 3). The difference was more pronounced in case of BMC (Fig. 3A) than BL (Fig. 3B).

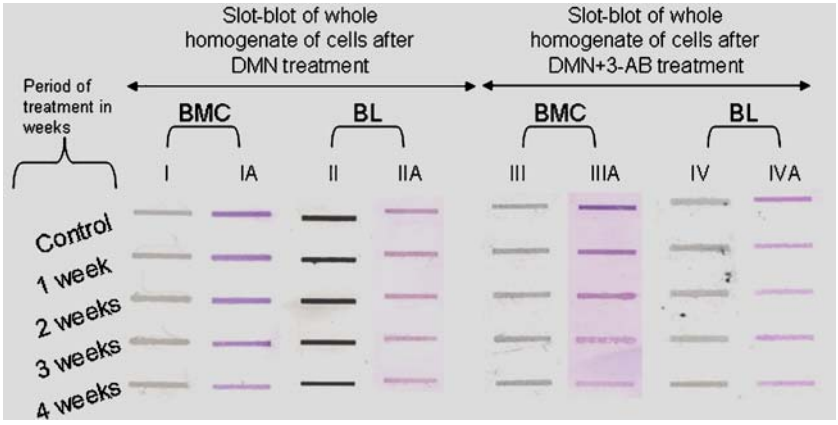


*Fig. 4*. Slot blots of whole homogenate of bone marrow cells (BMC) and blood lymphocytes (BL) of control and DMN or DMN+3-AB exposed mice. One of the slot blotted PVDF membranes of each experimental group was immunoprobed for total PAR of slotted proteins (right blots marked IA, IIA, IIIA & IVA) while their replica were stained with India ink for total proteins (left blots marked I, II, III & IV).

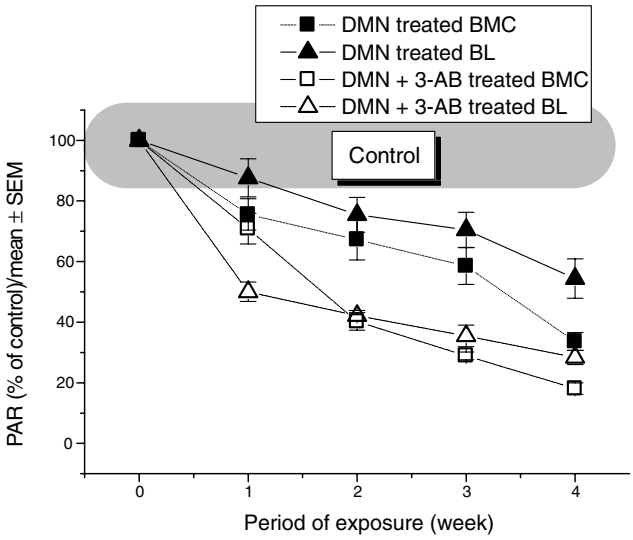


*Fig. 5*. Quantification plot of level of total PAR of all cellular proteins of bone marrow cells (BMC) and blood lymphocytes (BL) as a function of period of exposure to DMN alone (closed symbols) or DMN+3-AB (open symbols). The graph of mean intensity (pixel density) has been plotted as % of control after densitometric quantification of the immunodetected slot blots (Fig. 4). The level of PAR in age and sex matched control mice is shown as a shaded bar.

Even in the presence of 3-AB, PAR was still detectable in our investigation (Figs. 2 and 4). This is likely as 3-AB inhibits PARP enzyme, thereby, blocking further PAR of H1 protein. However, H1 protein was poly-ADP-ribosylated before 3-AB exposure was initiated. The persisting PAR is detected by the immunoprobe assay in our investigation. In principle, no PAR of H1 protein would be detected when it is totally stripped of PAR, as was shown earlier using a specific PAR degrading enzyme [10]. Physiologically, a PAR catabolizing enzyme, poly-ADR-ribose glycohydrolase, exists for removing PAR from target proteins [1]. The results (Fig. 3), therefore, suggest that rate of breakdown of PAR of histone H1 protein was faster in BMC (A) than BL (B) under the exposure conditions since the slope was higher for BMC.

The total cellular PAR detected by slot blot immunoprobe assay in both the tissues under identical treatment conditions gave a similar but clearer picture (Fig. 4). It is obvious that the slot blot immunoprobe assay detected PAR of all cellular proteins together, including that of histone H1. For equal amounts of proteins slotted in different experimental groups, the PAR of total cellular proteins exhibited duration of exposure dependent progressive lowering as evident by the quantitative plot (Fig. 5). In presence of 3-AB, the extent of lowering was more pronounced on expected lines (Fig. 5). There was no significant difference between the two tissues in terms of effect of DMN or DMN+3-AB exposure regimes (Fig. 5) as was noticed in case of H1 protein (Fig. 3). This suggests that PAR of other cellular proteins than histone proteins exhibited variable extents of poly-ADP-ribosylation in BMC or BL. Several high molecular weight cellular proteins, particularly PARP, are reported to be targets of PAR [1, 8–10]. Nonetheless, as a potential biomarker of initiation of carcinogenesis the total PAR of cellular protein, as detected by slot blot immunoprobe assay, seems to give a very clear picture whereby lowering of PAR was the hallmark of carcinogenesis [7–10]. This is in line with a recent study where inhibition of PARP was shown to increase genomic instability in cells exposed to genotoxic agents [22].

In conclusion, the findings further reaffirms our conviction that lowering of PAR, especially the total PAR, of BMC and BL proteins could be a biomarker of initiation of carcinogenesis by DMN in mice. Performing the slot blot immunoprobe assay, reported to be specific and sensitive [10],

is easy. Collection of blood samples is also least invasive. These considerations open up a likely possibility of use of slot blot immunoprobe assay of PAR in mass cancer screening program. Currently, investigation is on with human blood samples to test the feasibility of PAR as a biomarker of human cancer and its use in population cancer screening program.

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