STUDY OF INFLUENCE OF POLY-ADP-RIBOSYLATION AND REGRESSION OF CHEMICALLY INDUCED CARCINOGENESIS *IN VIVO*

BY

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I, **Brahmacharimayum Jaylata Devi,** hereby declare that the subject matter of this thesis is of the record done by me, that the content of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ institute.

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Dedicated to

My beloved Baba and Emma

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ABBREVIATIONS

°C	degree Centigrade
µg	Microgram
µl	Microlitre
3-AB	3-Aminobenzamide
Ab	Antibody
ADP	Adenosine diphosphate
Ag	Antigen
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
BMC	Bone marrow cells
BSA	Bovine serum albumin
cm	Centimetre
DMN	Dimethylnitrosamine
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme link immuno sorbent assay
Fig.	Figure
g	Centrifugal force
gm	Gram
KDS 1D	Kodak Digital Science 1D
lit.	Litre
M	Molar
mA	Milliampere
MAR	Mono-ADP-ribosylation
min^{-1}	per minute
ml^{-1}	per millilitre
mM	Millimolar
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NC	Nitrocellulose membrane
PAR	Poly-ADP-ribosylation
PARG	Poly-ADP-ribose glycohydrolase
PARP	Poly-ADP-ribose polymerase
PBS	Phosphate buffer saline
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second
SEM	Standard error of the mean
SPC	Sucrose potassium phosphate-$CaCl_2$ (buffer)
TBS	Tris buffered saline
TEMED	N, N, N', N'-Tetramethylethylenediamine
TTBS	Tween-20 tris buffered saline
UV	Ultra violet
V	Volts

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CHAPTER I

INTRODUCTION

1.1 ADP-RIBOSYLATION

ADP-ribosylation is a reversible post-translational modification of mainly chromosomal proteins (Althaus and Richter, 1987). The reaction is enzyme catalyzed. During the anabolic process, ADP-ribose moiety of a co-enzyme, nicotinamide adenine dinucleotide (NAD^+), is sequentially added onto acceptor proteins. Several *in vitro* analyses suggest that the modification of the target proteins by this reaction inactivate them (Shall, 1994). The catabolic process, on the other hand, successively degrades the ADP-ribose polymer from the target protein.

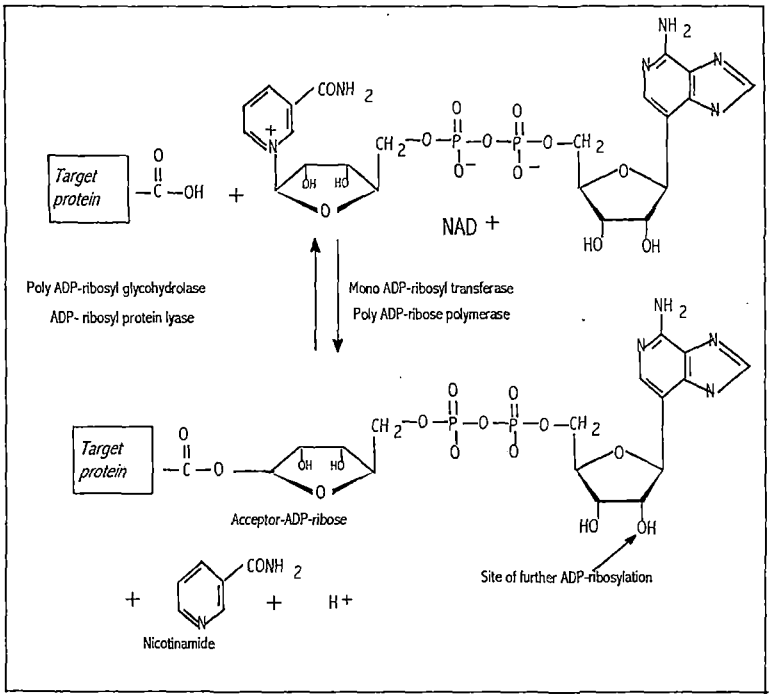

Figure 1: Schematic representation of ADP-ribosylation reaction.

In the primary reaction, ADP-ribose from NAD^+ is transferred to a specific amino acid on a particular protein generating mono ADP-ribosylated protein. The reaction is catalyzed by mono ADP-ribosyl transferases. The enzyme poly-ADP-ribose polymerase then adds further ADP-ribose residues from the substrate NAD^+ to form a dimer, which is then elongated by subsequent addition of ADP-ribose residues, thereby, forming linear and branched chains. The overall process is represented by Fig. 1.

1.1.1 Historical background

The historical background of the discovery of poly-ADP-ribosylation and its biological implications have been elegantly reviewed by Sugimura and Miwa, 1994. ADP-

ribosylation reaction was observed originally during the study of bacterial toxins, which demonstrated that diphtheria toxin inhibits mammalian protein synthesis (Strauss and Hendee, 1959). Like many scientific discoveries, the discovery of poly-ADP-ribose by Mandel, and his students at University of Strasbourg in 1966 was also accidental. This was initially interpreted as poly A polynucleotide. However, persistent and excellent graduate student, Doly (1968), checked out the results and demonstrated quite clearly that the product was a novel polymer associated with proteins. Two different Japanese groups (Fujimura *et al.,* 1967, Nishizuka *et al.,* 1967) later on confirmed this conclusion. Since then, several lines of investigations with improved biochemical techniques have broadened the understanding and scope of ADP-ribosylation reactions.

1.1.2 Mono-ADP-ribosylation (MAR)

When one ADP-ribose moiety is linked to a target protein, the process is regarded as mono-ADP-ribosylation (MAR). The reaction is not dependent on DNA. Moss *et al.,* 1990 have suggested that MAR might be part of the signaling mechanism for cell attachments or cell-cell contacts. In addition to this concept, Shall, 1995, has proposed the involvement of MAR reactions in several signaling systems associated with G-proteins, calcium levels, and cell surface interactions. The enzyme responsible for this reaction is mono-ADP-ribosyl transferase.

1.1.3 Poly-ADP-ribosylation (PAR)

In poly-ADP-ribosylation (PAR) reaction, a homopolymer of repeating ADP-ribose moieties is attached to a target protein. It is principally a nuclear reaction and hence, limited to eukaryotes. So PAR could not be detected in prokaryotic organisms. Approximately 95% of ADP-ribosylation reaction are in the form of PAR (Ueda, 1989). The reaction is catalyzed by enzymes poly-ADP-ribose polymerase (PARP) and mono-ADP-ribosyl transferase for biosynthesis. Poly-ADP-ribose glycohydrolase (PARG) and ADP-ribosyl protein lyase are enzymes involved in the degradative pathway. The overall reaction leads to formation of a branched or unbranched polymer, up to 200 monomers long on a target protein. The reaction is shown in Fig. 2.

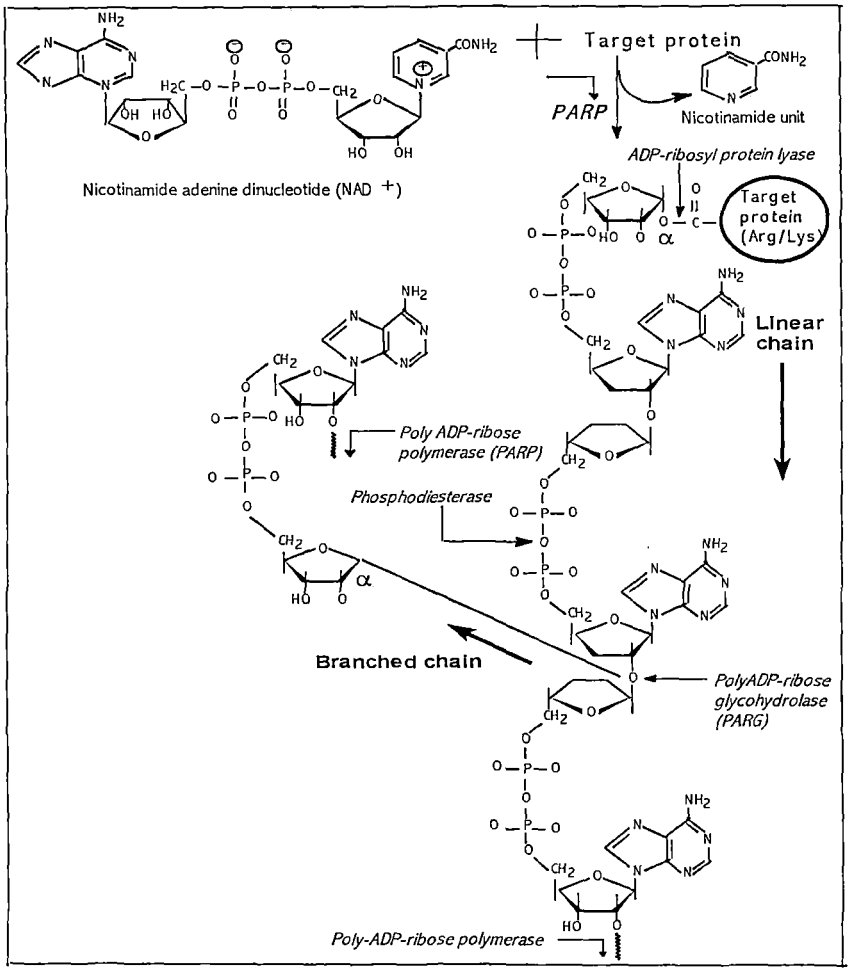

Figure 2: Poly-ADP-ribosylation reaction and enzymes involved in synthesis and degradation. PARP catalyses transfer and polymerisation of ADP-ribose residue from NAD^+ to protein carboxyl group. The same enzyme is responsible for growth of branched polymer. PARG is the main polymer-degrading enzyme.

1.2 CELLULAR DISTRIBUTION

Poly-ADP-ribosylation (PAR) reactions are ubiquitous in higher eukaryotes with exception to mammalian erythrocytes, which lose this activity during erythropoiesis consistent with the enucleation step (Althaus and Richter, 1987). PAR activity has been demonstrated in a number of plants (O'Farrel, 1995), in some lower eukaryotes and even in the dinoflagellate *Crypthecodium cohnii* (Werner *et al.*, 1984), an organism, which lacks histones and has a chromatin arrangement similar to prokaryotes. However, in yeast no PAR of proteins could be detected (Hayashi and Ueda, 1982). An analysis of the *in vivo* distribution of the PAR activity showed a preferential localization of the activity in polynucleosomal preparations (Giri *et al.*, 1978), whereas immunoelectron microscopy detected a preferential association of the synthetic enzyme to the nucleosomal core (Leduc *et al.*, 1986). Although the enzyme for PAR is primarily located in the nucleus, its

cytoplasmic counterpart has been found in the microsomal-ribosomal fraction of rat spermatogenic cells (Concha *et al.*, 1989). PAR activity has also been reported in ribosomes (Roberts *et al.*, 1975) and in mitochondria (Kun *et al.*, 1975).

1.3 TARGET PROTEINS AND AMINO ACIDS OF ADP-RIBOSYLATION

The known target proteins in eukaryotes for MAR include elongation factor elF_2, Gs and Gi subunits of adenylate cyclase, and transducin among other proteins (Miwa and Sugimura, 1990). For PAR, the known target proteins are nuclear proteins such as histones (Boulika, 1990), endonuclease (Tanaka *et al.*, 1984), topoisomerase I (Ferro and Olivera, 1982), reverse transcriptase (Buki *et al.*, 1991); DNA ligase II (Cressien and Shall *et al.*, 1982), and its synthetic enzyme, poly-ADP-ribose polymerase (Adamietz, 1987). The main amino acids in these proteins where ADP-ribose moieties get added include arginine (Takenaka *et al.*, 1995), cysteine (Jacobson *et al.*, 1990), asparagine (Sekine *et al.*, 1989) and diphthamide, a modified histidine (Iglewski and Dewhurst, 1991). Many different cellular nucleophiles have also been shown to serve as acceptors of ADP-ribose. The main eukaryotic enzyme preferentially catalyzes transfer of ADP-ribose moieties to glutamate and aspartate residues (Burzio, 1982) in proteins and to other ADP-ribose residues to generate polymers of ADP-ribose (Althaus and Richter, 1987). Free ADP-ribose can react non-enzymatically with lysine (Cervantes *et al.*, 1993) and cysteine (McDonald and Moss, 1993) residues.

1.4 ORGANISATION OF POLY-ADP-RIBOSE

Poly-ADP-ribose is a nucleic acid like homopolymer made up of repeating units of adenosine diphosphate ribose units. The primary structure contains ribose-ribose glycosidic linkages alternating with pyrophosphate bonds and with an adenine base attached to every second ribose. In addition, this unusual biopolymer may also be branched (Chambon *et al.*, 1966; Miwa *et al.*, 1981). The polymer is well characterized and it is structurally similar to a nucleic acid. Minaga and Kun (1983) have proposed a helicoidal conformaion for long ADP-ribose chains. Immunological studies suggest similarities between the secondary and tertiary structure of the polymer and single stranded DNA (Kanai and Fujimura, 1985). Branching points up to a proportion of 3%

(Alverez and Jacobson, 1987) can be found within the polymer (Kanai *et al.,* 1982) contrary to DNA and RNA which have none.

Each polymer residue carries two formal negative charges and an adenine ring capable of hydrogen bonding and hydrophobic interactions. Covalent binding of ADP-ribose polymers to nuclear proteins have long been studied (Althaus and Richter, 1987). Possibility of non-covalent interactions of ADP-ribose polymers with nuclear proteins, thereby, modulating chromatin structure has been suggested (Althaus *et al.,* 1995; Panzeter *et al.,* 1992; Saikia *et al.,* 1999; Schneeweiss *et al.,* 1995; Sharan *et al.,* 1998b). ADP-ribose polymers vary in complexity and may reach a length of more than 200 residues (Alvarez and Jacobson, 1987; Hayashi. *et al.,* 1983). The half-life of the polymer varies in relation to the length of the polymer chain as well as to the nature of the acceptor protein. After stimulation of PAR synthesis with alkylating agents, the half-life of the polymer is less than 1 min (Alverez and Althaus, 1989). The very short half-life of the polymer suggests the presence of very high turn over in intact cells. The natural content of poly-ADP-ribose in most tissues is rather low, compared to related polymers such as nucleic acids, and ranges between 3-30 ng ADP-ribose per mg DNA (Ueda and Hayashi, 1985)

1.5 ENZYMES INVOLVED IN ANABOLISM OF POLY-ADP-RIBOSE

Poly-ADP-ribose synthesis can be readily distinguished from DNA synthesis since DNA synthesis requires the presence of all four dNTPs and ATP, whereas the only nucleotide required for poly-ADP-ribose synthesis is NAD^+. Berger *et al.,* 1978 had demonstrated that the synthesis of the polymer has no direct effect on replicative DNA synthesis in progress. However, it is possible that the synthesis of the polymer could affect the structure or function of some of macromolecule. The followings are the enzymes involved in ADP-ribose biosynthesis.

1.5.1 Poly-ADP-ribose polymerase (PARP)

PARP is a ubiquitous enzyme present in high amounts in the nucleus of actively dividing cells and has been purified to homogeneity from a number of mammalian tissues (Althaus and Richter, 1987). The enzymatic activity is strongly enhanced by the presence of DNA strand breaks, both double (Benjamin and Gill, 1980) and single (Epe *et al.,* 1996). The

mechanism of the DNA strand breakage is probably related to abstraction of hydrogen atoms from the ribose of the DNA moiety, thereby opening the sugar ring (Szabo, 1998). A fast activation of PARP by signals evoked in the cell membrane, constituting a novel mode of signaling to the cell nucleus has been reported (Homburg *et al.*, 2000).

The PARP contains three functional domains (Kameshita *et al.*, 1984): a DNA binding domain located at the amino terminus; a central automodification domain that acts as an acceptor for PAR reaction; and a catalytic domain located at the carboxyl terminus. The enzyme performs three enzymatic steps (Althaus and Richter, 1987). The **initiation reaction** involves covalent attachment of an ADP-ribosyl moiety from NAD^+ to the amino acid residues of the target protein. **ADP-ribose chain elongation** causes attachment of ADP-ribose moieties on to protein bound ADP-ribosyl residues. And **branching reaction** introduces an ADP-ribose residue branching from a linear portion of the polymer.

1.5.2 ADP-ribosyl transferase

ADP-ribosyl transferases are found in viruses, bacteria and eukaryotic cells. The best-known mono-ADP-ribosyl transferases are extremely powerful bacterial toxins, which kill the host cells by inactivating crucial cellular targets via MAR. For example, cholera toxin and *Escherichia coli* heat-labile enterotoxin, pertussis toxin produced by *Bordetella pertussis*, exert their toxic action through MAR of a few specific enzymes or target proteins (Althaus and Richter, 1987; Ueda, 1989).

1.6 ENZYMES INVOLVED IN CATABOLISM OF POLY-ADP-RIBOSE

Poly-ADP-ribose catabolism is a complex situation involving many proteins and DNA. The quality of polymer, i.e. chain length and complexity, as well as preference for the nuclear substrate varies depending upon the availability of poly-ADP-ribose glycohydrolase (Laguex *et al.*, 1994). This reaction involves hydrolysis of the glycosidic (1"-2') linkages of poly-ADP-ribose to release ADP-ribose and mono-ADP-ribosyl-proteins (Tavassoli *et al.*, 1983). To date, three different enzymes, poly-ADP-ribose glycohydrolase, phosphodiesterase, and ADP-ribosyl protein lyase are known to involve in the catabolism of polyADP-ribose.

1.6.1 Poly-ADP-ribose-glycohydrolase (PARG)

PARG is a physiological counterpart of PARP. It hydrolyzes the branch points of poly-ADP-ribose (Miwa *et al.*, 1981). It is present in all eukaryotic cell types, except yeast, due to its antagonistic role with PARP (Lautier *et al.*, 1993). *In vitro* studies suggest that glycosidic action operates in biphasic, bimodal reaction mode (Hatakeyama, 1986). While polymer greater than 20 residues are degraded to smaller polymers in a fast and processive reaction, further degradation proceeds in a slowly distributive reaction mode. Braun *et al.* (1994) proposed three potential modes of action for PARG: exoglycosidic; endoglycosidic; and combination of both endo and exoglycosidic actions. Desnoyers *et al.* (1995) have recently demonstrated that presence of endoglycosidic activity of PARG could have a vital role to play in the involvement of PAR metabolism during DNA-repair and other cellular responses.

1.6.2 ADP-ribosyl protein Lyase

Very little is known about this enzyme. This enzyme cleaves the protein proximal ADP-ribose-glutamic ester bond (Okayama *et al.*, 1978). The elimination reaction catalysed by the lyase yeilds an unsaturated sugar identified as 5'-ADP-3"-deoxypent-2"-enofuranose (Oka *et al.*, 1984). The removal of the proximal ADP-ribose residue bound to the acceptor protein has been proposed to be the rate-limiting step in the catabolism of carcinogen-induced polymers (Wielkens *et al.*, 1982).

1.7 POLY-ADP-RIBOSYLATION (PAR) AND ITS INFLUENCE ON CELLULAR PROCESSES

Poly-ADP-ribosylation is reported to be one of the molecular events, which has links to various multiple cellular processes (Scneeweiss *et al.*, 1995; Shall, 1994; Sharan *et al.*, 1998a) including carcinogenesis (Boulika, 1991; Pariat, 1997; Miwa and Sugimura, 1990; Saikia, 1996). Some of the major events reported to be associated with PAR modification of different cellular proteins are schematically shown in Fig. 3 below.

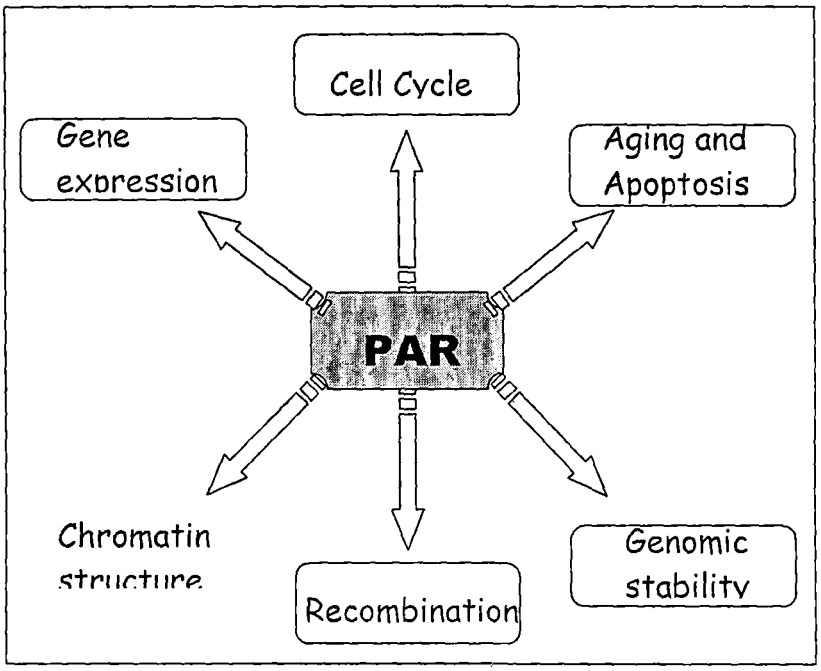

Figure 3: Schematic representation of various cellular processes related to PAR.

1.7.1 PAR and Chromatin structure

Nuclear DNA exists as a hierarchy of chromatin structures, resulting in compaction of the nuclear DNA about 10000-fold. The basic repeating unit in the chromatin is nucleosome. In 1997, Timothy Richmond and colleagues solved the crystal structure of the nucleosome core particle to a resolution of 2.8 Å. (Luger *et al.,* 1997). The higher order structure of chromatin is inaccessible to most nuclear processes such as DNA repair, replication and gene expression. These processes require, at first, undoing of higher order chromatin structure and, secondly, opening up of individual nucleosome. Each nucleosome is composed of a core histone octamer and a stretch of 167 bp of DNA wrapped twice around it. Unfolding of chromatin superstructure would require loss of specific contacts between the 1-72 amino acid segment of histone H1 and core histones at neighboring nucleosome (Boulikas, 1986). The PAR has been implicated in opening up of higher order chromatin structure *in vitro* (Niedergang *et al.,* 1985; Poirier *et al.,* 1982). It was further demonstrated that chromatin was unable to condense when histones (mainly H1) were poly-ADP-ribosylated using the endogenous polymerase (Aubin *et al.,* 1983).

The dramatic changes brought to chromatin structure even by a fraction of poly ADP-ribosylated H1 (less than 5% of the total) have been examined (Aubin *et al.,* 1983; Mathis and Althaus, 1987; Poirier *et al.,* 1982). Covalent linkage of ADP-ribose to histones H1 and other cores histones or to polymerase can result into transient dissociation of a core

histone octamer from DNA (Boulikas, 1990). It has been suggested that this dissociation might be mediated by the equal or higher affinity of H3 and H4 for poly-ADP-ribose than for DNA (Wesierska *et al.,* 1988). The dissociation of histones causes relaxation of chromatin superstructure. Poly-ADP-ribose might then be rapidly degraded by PARG allowing for the reassociation of the histones to DNA. This concept is strongly supported by the result that PARG was found to compete with PARP for PAR reaction in an *in vitro* system (Thomassin *et al.,* 1992). PARP then could modify histones at adjacent nucleosomes or so on. PAR, is therefore, involved in both the unfolding of higher order chromatin structure as well as unfolding of individual nucleosomes. Mono-, oligo- or poly-modified H1, core histones or polymerase might have different roles in altering chromatin and nucleosome structure. Further degradation of poly ADP ribose on H1 by purified PARG restored the native condensed chromatin superstructure (de Murcia, 1988). PAR also influences structural organisation of chrmosomal DNA by subjecting to external stimuli and radiation (Schneeweiss *et al.,* 1995; Sharan *et al.,* 1998a). The interaction of ADP-ribose polymers of automodified PARP *in vivo* with adjacent proteins predominantly histones, may provoke alterations in chromatin conformation through noncovalent interactions with histones (Panzeter *et al.,* 1992).

1.7.2 PAR and cell cycle

Since PAR of histones influence condensation and decondensation of chromatin superstructure, its involvement in cell cycle regulation is likely. Adolph (1987) investigated PAR of nuclear proteins and its influence on cell cycle. In HeLa cells more than 100 acceptor proteins were detected in the interphase nuclei while in metaphase only PARP itself served as acceptor protein. The activity of PARG was doubled during G_1 phase (Tanuma and Otsuka, 1991). Schreiber *et al.* (1995) had shown the activity of PARP in G_2 + M phase in HeLa cells lacking PARP function. PARP mRNA level during cell cycle was analyzed and found to culminate in the G_1 phase (Thiebodeau *et al.,* 1989). A possible cell cycle dependent biosynthesis of PARP has also been proposed for mouse SV 40-3T3 cells (Sooki-Toth *et al.,* 1987). PARP seems to be critical for the induction of G_1 arrest and is involved in the regulation of G_2 arrest (Masutani *et al.,* 1995). The expression of PARP cDNA in yeast cells, lacking PARP and PARG activities, resulted in poly-ADP-ribose synthesis, which provoked cell cycle retardation. This resulted in a specific delay of the G_1 phase, decreased cell viability in stationary cultures, and an increased sensitivity to radiation (Avila *et al.,* 1994; Collinge and Althaus, 1994).

1.7.3 PAR in gene expression and cell differentiation

PAR reactions also play an important role in the regulation of gene expression and cell differentiation (Nagao *et al.,* 1991, Smulson *et al.,* 1995). Several investigations using inhibitors of enzyme PARP have suggested that PAR reactions may regulate gene activity (Leverence, 1988; Ghani, 1983). The role of PAR in gene expression seems to be both at transcriptional and translational level. Many studies show that the enzyme PARP is associated with the active regions of transcription (de Murcia *et al.,* 1988; Hough *et al.,* 1994). The transcriptionally active gene domains are hypersensitive to the action of nucleases (Reeves, 1984). *In vitro* experiments involving PARP in transcription showed that PARP could suppress nonspecific transcription induced by single strand breaks in DNA (Kurl *et al.,* 1985). This property would seem to be associated with the binding PARP to DNA and not to the catalytic activity of PARP (Ohsuki *et al.,* 1984). The PARP by binding to the breaks would permit the precise transcription of genes by preventing transcription initiation at the breakage points in DNA. Work done by Kun's group demonstrated that besides the indiscriminate binding of PARP to DNA termini or to single strand breaks, the enzyme can associate to internal regions of DNA molecules showing bent or cruciform structures (Sastry *et al.,* 1990). Drugs interacting with PARP inhibited carcinogen induced cellular transformation (Kun *et al.,* 1983), prevented tumorigenesis (Tseng *et al.,* 1987) and led to an altered chromatin structure resulting in gene amplification (Burkle *et al.,* 1987). The relevance of PARP in transcription regulation is supported by the observation that in cells of PARP negative mice proliferation following γ irradiation was impaired (Wang *et al.,* 1995).

The activity of PARP appears to be regulated on a posttranslational level (Bhatia *et al.,* 1990; Herzog *et al.,* 1989; Sharan *et al.,* 1996; 1998b). A potential role of PARP in cell differentiation processes has been suggested. Cornelissen *et al.* (1985) had shown the delay of morphological switch of the parasites surface proteins when treated with 3-aminobenzamide. It has been reported that PAR is required for the initiation of differentiation (Taylor and Williams, 1988). The activity of PARP is controlled by regulation of protein levels during differentiation of human leukemia and neutrophilic cells (Bhatia *et al.,* 1995).

1.7.4 PAR in DNA damage and repair

Involvement of polyADP-ribose in DNA excision repair has been suggested by various lines of evidence (Althaus and Richter, 1987; Shall *et al.*, 1994). For the molecular mechanism of this involvement, Durkacz *et al.* (1980) suggested that inhibition of polyADP-ribose synthesis retarded the rejoining of damaged DNA. Lindahl *et al.* (1995) revealed that PARP is not a necessary repair enzyme, but is able to stimulate the excision repair pathway. In contrast to conclusion deduced from the histone-shuttle model (Althaus, 1992). Satoh and Lindahl (1992) proposed a model of a histone independent participation of PARP in the excision repair pathway. Polymer synthesis during DNA repair was investigated in an *in vitro* system (Satoh *et al.*, 1994). PARP knock-out mice showed no drastic decrease in their DNA repair capability (Wang *et al.*, 1995), implying that PARP is not directly participating in DNA excision repair. The involvement of PARP in the DNA synthesis step of the base excision repair process has been reported (Dantzer *et al.*, 1999).

1.7.5 PAR in ageing and apoptosis

Grube *et al.* (1992) observed a positive correlation between life span and PARP activity of 13 different species. Long living species had higher PAR activity. The relationship between PAR and apoptosis, a programmed cell death originating within, in response to mild damaging stimuli (Kerr *et al.*, 1991) has been reported (Scovassi and Poirier, 1999). It has been suggested that high PAR reaction prevent genetic alterations. Application of DNA damaging cytotoxic chemicals led to apoptosis (Marks and Fox, 1991). Cell lysis was found to be associated with the activation of PARP (Schrauffstatter *et al.*, 1986). Induction of apoptosis and level of PAR of cellular protein and nuclear proteins in mouse *ex-vivo* cells following γ irradiation has been examined (Humtsoe, 2000). The result, on the other hand, showed that apoptosis was measured maximum at about 2 Gy which coincided with the dose at which PAR of histones was inhibited. It is proposed that nitric oxide induced neurotoxicity, caused by NAD^+ depletion due to stimulated PARP activity, resulting in cell lysis (Zhang *et al.*, 1994). The enzyme PARP seems to be activated at the time the DNA ladder appears and its activation is, therefore, an excellent marker for apoptosis (Lazebnik *et al.*, 1994). Based on recent studies using PARP knocked-out cells, several groups concluded that PARP is important for genomic stability but dispensable for apoptosis (Wang *et al.*, 1997).

1.7.6 PAR in recombination and maintenance of genomic stability

Eukaryotic cells contain substantial amounts of repeated DNA sequences. During recombination events DNA breaks appear. PARP is able to bind rapidly and tightly to such breaks, suggesting a possible participation of this enzyme in recombination processes. Farzaneh *et al.* (1988) observed that PARP stimulates the integration of donor DNA into the host genome during DNA transfection in eukaryotes. Inhibition of PARP by 3-aminobenzamide led to an increase of recombination events in several animal and plant cell systems, as shown by an enhanced sister chromatid exchange frequency (Oikawa *et al.*, 1980). It has been reported that poly-ADP-ribose synthesis in the vicinity of DNA strand interruptions causes a negative charge repulsion between the polymer and the DNA, probably to prevent accidental homologous recombination within tandem repeat DNA sequences (Satoh *et al.*, 1994).

PARP is involved in cellular defense mechanisms against genotoxic agents. Shall, 1994 showed decrease in the cellular levels of NAD^+ due to biosynthesis of poly-ADP-ribose after treatment with alkylating agents. This decrease was not observed with cytotoxic agents lacking genotoxic effects (Althaus and Richter, 1987) nor with genotoxic agents used in the presence of PARP inhibitors (Jacobson *et al.*, 1980). The nature of alterations in the genome is proposed to activate PARP (Benjamin and Gill, 1980; Miller and Miller, 1975). From these observations a regulatory role of PAR in recombination and maintenance of genome has been suggested.

1.8 CARCINOGENESIS

The normal development and growth of every living organism is under well defined and precise mechanisms regulated within the organism itself. The normal cells proliferate in response to an array of external, mostly locally produced, growth factors produced by one cell type to activate a second. These growth factors exert their proliferative action after binding to appropriate receptors and induce a cascade of responses most of which involve phosphorylation events. When certain factors introduce defects in normal homeostatic control mechanisms, the cells escape controls on cell birth, death, senescence and interactions with other cells (Bertram, 2001). They either dedifferentiate or continue to divide without any control (anaplasia) and acquire characteristics of stem cells (hyperplasia). Uncontrolled cell proliferation and local tissue invasion and/ or distant

spreads (metastasis) are the main characteristics of these transformed cells. Eventually, a number of such abnormal cells accumulate leading to a cancerous/tumor mass (Weinberg, 1983). Tumor cells have found mechanisms to enable constant activation of these proliferative signals. Thus, cancer is characterized by dedifferentiation, uncontrolled cellular growth with local tissue invasion and/or systemic metastasis.

Carcinogenesis is complex multistep process involving multiple genetic alterations in somatic cells. These alterations include mutations, rearrangements and loss of gene function and amplification of genes (Pitot, 1986). It has been suggested that loss of growth control as well as inactivation of the apoptosis pathway are central for survival and proliferation of tumors during the *in vivo* process of multi-step tumorigenesis (Gill and Fisher, 1997). Cancer cells display an abnormal variability in the size and shape of their nuclei and in the number and structure of their chromosomes. Indeed abnormal nuclear morphology is one of the key features used by pathologists to diagnose cancer.

The process of carcinogenesis is categorized into three distinct steps.
Initiation: Cellular genome is irreversibly altered during this process.
Promotion: Partially reversible increase in monoclonal expansion of the transformed or initiated cell population.
Progression: Irreversible step involving demonstrable alterations in the cell genome by conversion of promoted cells to transformed cells.

The stepwise changes establish a tumor at the end. A tumor can be either benign or malignant. Benign tumors are proliferating cell population that neither invades surrounding tissues nor metastasize but remain encapsulated by a well-defined fibrous cover. It exhibits lesser degree of anaplasia (loss of structural organization and useful function), usually grow slowly and is less dangerous. Malignant means to have property of local invasion, destructive growth and metastasis. The malignant cancers are almost never encapsulated, have greater degree of anaplasia and exhibit autonomy (ability to grow unrestrained manner in the host). Genetically cancer cells are unstable and recurrence is common.

Among the main factors causing cancers, different types of radiation, viruses, other environmental and genetic factors and chemicals are implicated (Ames and Gold, 1991). A

hypothesis suggests that some genetic alterations act on genetically determined predisposition and results in the unregulated proliferation of cells (Dorr and Koeller, 1994). It is widely accepted that mutation or degradation of the genes playing roles in the integrated control of growth and differentiation may lead to the generation of malignant neoplasias. These genes are termed as protooncogenes and are present in all higher cells (Cooper and Lane, 1984). Their activation to oncogenes by transduction, translation, insertional mutagenesis, deletion, amplification and point mutation may induce quantitative over expression and generally confers to oncogenes a transforming capacity. Oncogene activation by carcinogens, radiation or other events is necessary for cancer initiation (Cooper and Lane, 1984).

Induced carcinogenesis is that resulting from exposure of organisms to carcinogenic agents such as ionizing irradiation, ultraviolet light or various chemicals. Carcinogenic effect of both naturally occurring and man made carcinogens have been observed (Sharan and Wary, 1992; Sharan, 1996).

1.9 CHEMICAL CARCINOGENESIS

Chemical carcinogens are usually reactive intermediates, as electrophilic reactants or radical cations that can interact with cellular macromolecules especially the DNA. They can be defined operationally by their ability to increase the occurrence of neoplasm. The most frequent chemical reaction giving rise to DNA damage can be characterized as an electrophilic attack upon a cellular nucleophile (Miller and Miller, 1975). Depending on their mode of action, chemical carcinogens are of two types. **Genotoxic** that interact with and alter DNA and **epigenetic** that comprises those chemicals for which no evidence exists of direct interaction with genetic material, but which produce another biological effect that could be the basis for their individual carcinogenicity.

In 1964, Brookes and Lawley showed carcinogenic potency of polycyclic aromatic hydrocarbons, which provided the impetus for many subsequent studies on the molecular basis of chemical carcinogenesis. With the growing realization that some human cancers have an environmental origin that could be linked directly to chemical exposure, the list of carcinogenic chemicals rapidly expanded (Doll and Peto, 1981; IARC, 1987). Most known chemical carcinogens react chemically with DNA, either directly or as derivatives

produced by metabolic activation to chemically reactive electrophilic species, which can then form covalent adducts (Singer and Grunberger, 1983).

1.9.1 Dimethylnitrosamine (DMN) induced carcinogenesis

Chemical carcinogen, DMN has been chosen for this work. It is a nitroso derivative of aliphatic hydrocarbons and is the simplest member of the class nitrosamine and is a known hepatocarcinogen (Pariat, 1995). Nitrosamines are chemically stable compounds under physiological conditions. Following administration to animals, they are metabolized to generate reactive products which are probably electrophilic alkylating agents (Montessano, 1982) Biological activity of the reactive products leading to carcinogenesis begins with alkylation at certain sites in DNA. These sites include N-1, N-3 and N-7 sites of adenine, N-3, N-7 and O-6 of guanine, N-3 and O-2 of cytosine, N-3, O-4 and O-2 of thymine and the phosphate groups. Depending on the efficiency of the repair processes in the target cells/tissues, the extent of the carcinogenicity and the mutagenicity are determined. Nitrosamines have been reported to be complete carcinogens and can act as initiators and promoters (Wigley *et al.,* 1985). This is corroborated by the result that carcinogenesis was induced *in vivo* in mice causing alteration in the activity of PARP enzyme under its influence (Pariat, 1997, Pariat and Sharan, 1998). Various *in vivo* and *in vitro* studies show that N-nitrosamine induced adduct formation on oxygen molecules, has more potential to cause transformation than on other sites (Montessano, 1982). They produce usually systemic tumors distant from the site of application. Nitroso compounds have also been shown to be toxic (Magee and Barnes, 1967), teratogenic (Druckrey, 1973), mutagenic (Montessano and Bartsch, 1976) and clastogenic (Kihlman, 1966). The carcinogenic potential of DMN was discovered serendipitously when animals surviving acute doses later were found to develop liver carcinomas (Magee, 1972).

1.9.1.1 Toxicity

A single dose of about 25 mg/ kg DMN administered orally, to the rat, or by intravenous, intraperitoneal or subcutaneous injection produced serious destruction of liver tissue accompanied by hemorrhages into the liver and lungs. Often there occurred a serious accumulation of fluid in the abdominal area and blood in the lumen of the intestines. Death usually occurred in 2-4 days or the animal recovered completely. Rabbits, mice, guinea pigs, and dogs all developed similar liver damage (Magee and Barnes, 1967). On the other hand, continuous low dose exposures also cause cancer of the liver and higher

concentrations for short periods (or as a single dose) result in kidney tumors (Magee and Barnes, 1967).

1.9.1.2 Metabolic activation

Dutton and Heath (1956) first demonstrated metabolism of DMN in rat and mouse. DMN undergoes a metabolic activation to exert their carcinogenic effect. This activation process occurs through α-hydroxylation that generate an active electrophile- a methylcarbonium ion CH_3- after a sequence of biochemical reactions shown in the scheme drawn here (Fig. 4). The methylcarbonium is able to react with cellular macromolecules producing toxic and carcinogenic effects. It was discovered that 0-6 methylguanine was a product of reaction of activated nitrosamines with DNA and that this base, if not repaired, could introduce point mutations which were potentially carcinogenic (Bartsh and Montessano, 1984; Lawley, 1980). Microsomal mixed-function oxidase is solely responsible for the metabolic activation of DMN.

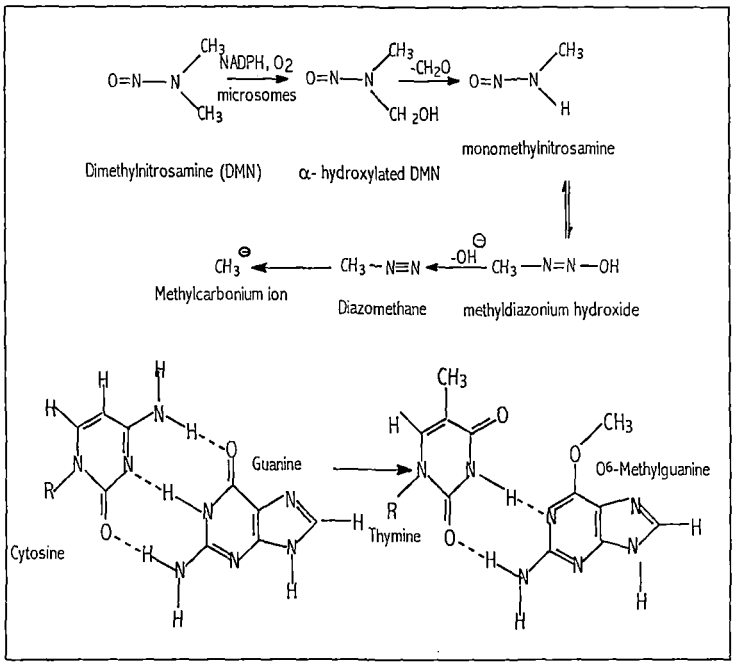

Figure 4: Metabolic activation of dimethylnitrosamine and reaction product with DNA.

However, it is important to emphasize strongly that other metabolic degradation pathways are known that are usually considered to lead to detoxified metabolites. Both reaction types may proceed either concurrently or sequentially. The balance of activation and detoxication mechanisms determines the biological effect of DMN.

1.10 DALTON'S LYMPHOMA ASCITES INDUCED TUMORIGENESIS

Ascites can be defined as peritoneal exudate, which includes fluid and inflammatory cells. The tumor, Dalton's lymphoma, is maintained in ascites form in the peritoneal cavity of Swiss albino mice. Dalton's lymphoma originated in the thymus gland of DBA/ 2 mouse at National Institute, Bethesda, MD, USA in 1947. Subsequently, an ascites form was developed by repeated intraperitoneal (i. p.) transplantation of the tumor (Goldie and Felix, 1951). An outstanding characteristic of the growing tumor is its capacity to elicit production of a new capillary endothelium from the host (Alguire, 1947). Cell growth is the fundamental feature of various stages of experimental carcinogenesis. Ascitic cells are generally rounded or convex, have reduced adhesion to substratum, infinite life span, loss of contact of inhibition of movement and multi layering in culture.

1.11 3-AMINOBENZAMIDE

Inhibitors of poly-ADP-ribose metabolizing enzymes would be useful for studies on the physiological significance of poly-ADP-ribose reaction in regulation of nuclear functions. Purnell & Whish (1980) reported that the substituted benzamides such as 3-methoxybenzamide and 3-aminobenzamide (3-AB) are potent and specific inhibitors of PARP. Common features of these inhibitors are presence of a carboxamide group built in a polyaromatic heterocyclic skeleton (Banasik and Ueda, 1994) At high levels of DNA damage their effect is to significantly reduce enzyme activity rather than completely block it (Sims *et al.,* 1982).

3-AB is a well-characterized competitive inhibitor of PARP with relatively low Ki (Durkacz *et al.,* 1980). It has been found to be effective on PARP at low doses, while higher concentrations are required to inhibit mono (ADP-ribosyl) transferases (Rankin *et al.,* 1989). It is reported to be involved in numerous subcellular processes including cell death and has been found to induce cytoskeleton rearrangement (Malorni *et. al,* 1994). Also suggested to be a versatile chemical capable of exerting antiproliferative, cytostatic as well as cytotoxic and anti apoptotic effects (Tiozzo *et al.,* 1996).

It has been reported that when a different range of 3-AB concentrations was used paradoxical results were obtained (Cleaver *et al.,* 1985). Up to 1 mM, 3-AB appeared to

reduce DNA strand break frequencies in cells damaged by methyl methane sulfonate. Doses of 1 mM, on the other hand, increased break frequencies and caused 88% inhibition (Banashik *et al.,* 1989). The 2 mM concentration of 3AB was reported to cause 96% inhibition of PARP activity (Sims *et al.,* 1982). At high concentrations, many non-specific side effects and cellular toxicity predominated (Cleaver *et al.,* 1985).

1.12 PAR AND CARCINOGENESIS

A cascade of molecular events drives a normal cell to a cancerous cell during the complex multistep process of carcinogenesis. The earlier events in the initiation phase include alterations in gene expression pattern, expression of neogenes, shutdown of differentiation genes, etc. Occurrence of these events involves change in the structural organization in chromatin (de Murcia *et al.,* 1988). As has been discussed earlier, PAR of chromosomal proteins significally mediate interaction of these proteins with DNA, thereby, exerting influence on structural organization of chromatin.

A direct relationship between the relaxation of polynucleosome structure and PAR has been shown (de Murcia, 1988; Saikia *et al.,* 1999; Schneeweiss, *et al.,* 2000; Sharan *et al.,* 1996; 1998a). The role of PAR in modulating chromatin superstructure suggests a possible influence of PAR reaction in carcinogenesis.

The involvement of ADP-ribosylation in cell transformation was first suggested from the observation that malignant or transformed cells *in vitro* have higher poly-ADP-ribose polymerase activity than the normal or untransformed cells (Burzio *et al.,* 1975; Leiber, 1973). PAR is also implicated in carcinogenesis during initiation and promotion stages (Boulikas, 1991). Studies on cells in culture and on animals demonstrated that inhibitors of PARP potentiate the cytotoxicity of carcinogens and enhance or inhibit tumor growth (Boulikas, 1991). It has also been suggested that malignant transformation is associated with changes in regulation and expression of genes caused by PAR dysfunction (Borek *et al.,* 1984).

Since carcinogenesis is a multistep process consisting of initiation, promotion and progression (Pitot, 1986), the effects of inhibitors on the various stages of carcinogenesis may differ. The initiation step may be related to genetic alteration, point mutation,

rearrangement and amplification of proto-oncogenes or suprression of tumor suppressor gene like p53 (Bertram, 2001). In this step involvement of PAR reaction is possible since molecular events related to this stage have strong influence by chromatin architecture. There are reports on the involvement of tumor promoters in induction of sister chromatid exchanges (Kinsella, 1978) and gene amplification (Varshausky, 1981). ADP-ribosylation might be related to promotion, since nicotinamide increased the incidence of diethylnitrosamine initiated renal tubular cell tumors in rats (Rosenberg, 1985). The progression step is proposed to be associated with amplification of oncogenes. Amplification of the integrated SV40 DNA sequence by MNNG was enhanced by 3aminobenzamide, inhibitor of PARP, in Chinese hamster cell line (Burkle, 1987). This suggests that PAR may also be involved in tumor progression.

In general it has been observed that the inhibition of PARP, inevitably lead to inhibition in the repair of lesions in the DNA. Thus, enhancement in the preferential killing of cancer cells over normal cells by the combined action of poly-ADP-ribosylation inhibitors with carcinogens, compared with the effect of the carcinogen alone, may be caused by blockage in repair (Boulikas, 1992). Cancer cells, due to their rapid growth, will be damaged more than quiescent cells by carcinogens that interfere with DNA synthesis. On the other hand, the induction of tumors in animals or the increase in transformation frequency of cells in culture may arise from inhibition in repair in normal cells after treatment with inhibitors and carcinogens (Boulikas, 1992). Cancer may arise by over expression of specific oncogenes. Malfunctions in DNA repair mechanism promote carcinogenesis. In addition, a large number of DNA rearrangements move inactive genes into active chromatin domains and vice versa, resulting in their activation or inactivation. This phenomenon, known as position effect variegation (Boulikas, 1992), often occurs in tumor cells and is promoted by strand breaks. In these events also PAR might be involved.

AIMS AND OBJECTIVES

This investigation was designed to detect metabolic level of PAR *in vivo* under different conditions. Considering the limitation of conventional isotopic assay of cellular PAR, the work envisaged development and optimization of an immunoassay for PAR using anti poly- ADP-ribose polymer antibody. To get insights into the biological aspects, an experimental hepatocarcinogenesis model in mice was proposed to be used. Study was

planned for the initiation stage of carcinogenesis only as it is the most critical and irreversible step in the process. In addition, an ascites tumorigenesis experimental model was also proposed to be used for comparison. The following aims were and objectives were set forth:

1. Development and optimization of an immuno assay for measurement of cellular PAR.
2. Quantification of levels of PAR of total cellular proteins in liver, spleen cells and BMC during cellular transformation induced by DMN and during Dalton's lymphoma ascites induced tumorigenesis in mice.
3. Quantification of levels of PAR of individual histones from liver and spleen cells during cellular transformation and tumorigenesis.
4. Finding correlation between PAR and carcinogenesis process.
5. Study of status of chromatin superstructure during cellular transformation.
6. Study of effects of 3-aminobenzamide on PAR during cellular transformation.

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CHAPTER II

MATERIALS AND METHODS

2.1 CHEMICALS

Indigenous chemicals of analytical grade obtained from various indigenous sources were used unless otherwise mentioned. Acrylamide, bis-acrylamide, ethylenediaminetetra acetic acid (EDTA), ammonium persulfate, dimethylnitrosamine, 3-aminobenzamide, agarose (type I: low EEO), (N-[2-hydroxy ethyl] piperazine-N-[2-ethane sulphonic acid]) (HEPES), DNA type 1: sodium salt from calf thymus, DNase I, ethidium bromide, TRIS (hydroxymethyl) methylamine, and dithiothreitol (DTT), N,N,N′,N′-tetramethylethylenediamine (TEMED), ß-mercaptoethanol, PBS tablets, bovine serum albumin (BSA), polyoxyethylene-sorbitan monolaurate (tween-20), coomassie brilliant blue R, coomassie brilliant blue G, sephadex G-25, alkaline phosphatase conjugated goat anti-rabbit IgG, nitrocellulose membrane: immobilon-NC (0.45 µm) and Sigma fast BCIP/NBT buffered substrate tablet were obtained from Sigma Chemical Co. (USA). Calibration proteins for SDS-poly acrylamide gel electrophoresis (Mr 14000-170000) were purchased from Boehringer Mannheim (Germany) while hybond-C extra supported nitrocellulose membrane (0.45 µm) from Amersham International Inc. (England) and staining ink from Rotring-werke Riepe KG, (Germany) were used.

2.2 INSTRUMENTS

The following instruments were used for carrying out the experiments, and the analyses of data:

DU-60 Spectrophotometer (Beckman); Microscope (Carl Zeiss JENA 30-G0603); Gel electrophoresis apparatus GNA-100 (Pharmacia); Electrophoresis power supply 200/2.0, Mini-transilluminator, Bio-dot® SF microfiltration apparatus, Mini-PROTEAN® II electrophoresis cell, Mini trans-blot® electrophoretic transfer cell, Gel dryers model 543 with vacuum pump and vapor trap, Imaging Densitometer GS-690 with molecular analyst PC software 1.5 (all Bio-rad); Digital zoom camera DC120 with 1D image analysis software (Kodak digital science™) attached to zenith PC LR4Dc; Centrifuges: Varifuge 20 RS (Heraeus sepatech), SPINWIN microcentrifuge (Remi), Microcentrifuge 5414 S (Eppendorf); Weighing balances (Sartorius, METTLER TOLEDO), Cyclomixers, Magnetic stirrers, Water-bath shaker, Tissue homogenizer, Incubators, and Rotary vacuum pump, Lyophilizer (all Remi).

2.3 ANIMALS

Swiss albino mice (Balb/c) were obtained from Assam Veterinary Biological, Guwahati. Its inbred line was maintained in communal cages in a well-ventilated animal room under controlled temperature (22 ± 2°C) with 12 h light and 12 h dark conditions. They were provided with standard mouse diet (Pranav Agro Industries Ltd., Delhi) and tap water *ad libitum*. The animals of same sex, aged 2-3 months and weighing about 25-30 g, were used in all experiments. Russian Chhinchilian strain rabbit, for raising polyclonal antibody, was purchased from Indian Council of Agriculture Research, Barapani, Meghalaya.

2.4 CARCINOGEN ADMINISTRATION

Dimethylnitrosamine (DMN) solution was prepared in water at a dose rates of 20 mg kg^{-1} and 10 mg kg^{-1} body weight. To separate groups of mice the carcinogen solutions were administered in drinking water *ad libitum*. Chronic treatment continued till 8 weeks. Among the animals receiving 20 mg kg^{-1} body weight dose, 77 % died usually during 4-6 weeks period of treatment. So the dose was considered lethal and was not continued for experimental purpose. The animals receiving 10 mg kg^{-1} body weight dose were used for the experiments. The observations were made at 2, 4, 6 and 8 weeks. The animals were provided with regular food supply during the treatment period. Age matched unexposed animals served as controls for all experiments.

2.5 3-AMINOBENZAMIDE (3-AB) ADMINISTRATION

3-aminobenzamide solution was prepared in double distilled water at a concentration of 10 mg kg^{-1}. To separate batches of mice intraperitoneal (i.p.) injections of 3-AB, at a dose rate of 5.45 mg kg^{-1} body weight (equivalent to 2 mM 3-AB per injection) per week was administered either alone or in conjunction with the carcinogen, DMN.

2.6 DALTON'S LYMPHOMA

2.6.1 Source and maintenance of the tumor

Dalton's lymphoma was originated in the thymus gland of DBA/2 mouse at National Institute, Bethesda, USA in 1947. Subsequently an ascites form was developed by repeated intraperitoneal (i. p.) transplantation of the tumor (Goldie and Felix, 1951). The ascites Dalton's Lymphoma was a generous gift from Cytogenetics laboratory of Zoology Department, N. E. H. U., Shillong. The tumor was maintained *in vivo* in inbred mice by serial intraperitoneal (i.p.) transplantation into the abdominal cavity of each mouse. Each transplant consisted of 10^7 cells suspended in 0.25 ml in 0.001 M PBS containing 0.138 M NaCl and 0.0027 M KCl; pH 7.4 at 25 °C. The tumor transplanted animal normally survived for 21-23 days. For the present study the transplantation was from a 15-day old donor ascitic mouse. Experimental observations were made on alternate days up to 15 day from the first day of transplantation.

2.6.2 Preparation of lymphoma ascites cells

Ascites cells were taken out from the abdominal cavity of a 15-day old tumor-bearing donor in an eppendorf tube by a sterile syringe. Red blood cells present were lysed using 0.85 % (w/v) ammonium chloride solution. The cell suspension was centrifuged at 2,000 x *g* for 5 min. The pellet of ascitic cells was suspended in PBS.

2.6.3 Cell counting

The number of cells in the suspension was determined by visual counting of cells on a Buerker cell counting chamber using a phase contrast microscope. Usually, a 200 x diluted cell suspension was used for the counting and number of cells was counted in 10 squares. The number of cells was calculated using the following equation:

Number of cells ml^{-1} = average cell number per square x dilution factor x 10^4

2.6.4 Transplantation of ascites cells

To a group of animals of same sex and age, 10^7 ascites Dalton's lymphoma cells were intraperitoneally transplanted into the abdominal cavity of each mouse. The mice were given normal food and drinking water *ad libitum*.

2.7 ISOLATION OF ADP-RIBOSE POLYMER

Heterogeneous ADP-ribose polymers were isolated following as described earlier (Saikia, 1996). Briefly, normal Swiss albino mice were killed by cervical dislocation, spleens were excised and cell suspension was made after the cells were squashed out in 1 ml of pre-chilled PBS. The cell suspension was homogenized and centrifuged at 1,300 x *g* for 15 min at 4°C. The cell pellet was collected, resuspended in 10 % TCA and incubated for 30 min on ice. After centrifugation at 20,000 x *g* for 15 min the pellet containing protein-bound endogenous poly-ADP-ribose moieties was washed with water to remove buffer and then dissolved in 0.1 M Tris-glycine buffer, pH 10.5 with occasional vortexing. It was again centrifuged at 20,000 x *g* for 60 min after incubating at 37 °C for 60 min with regular gentle shaking. The supernatant fraction containing the ADP-ribose polymers was desalted on Sephadex G-25 gel by elution with 0.03 M Tris-barbiturate buffer, pH 7.2 containing 6 M urea and 0.1 % SDS at a flow rate of 1 ml per 5 min. The eluent fractions of the peak were pooled and dialyzed extensively for about 48 h in double distilled water. The dialysate was frozen, lyophilized and stored for use as an antigen. Protein, DNA, and RNA concentrations were checked by standard methods of Bradford, Diphenylamine, and Orcinol, respectively (see Sections 2.19, 2.20, 2.21).

2.8 RAISING POLYCLONAL ANTIBODY AGAINST POLY-ADP-RIBOSE

2.8.1 Immunization of rabbit

Specific antibody against ADP-ribose polymer was raised in a healthy young rabbit in the laboratory. For the priming immunization the lyophilized antigen, poly-ADP-ribose, was dissolved in PBS, pH 7.4 at a concentration of 2 mg ml^{-1}. It was further diluted at 1:4 ratio with PBS to make the final concentration 0.4 μg μl^{-1}. Emulsion of the polymer was prepared with equal volume of Freund's complete adjuvant (FCA) by homogeneous mixing. The resulting thick, stable emulsion of ADP-ribose polymer and FCA in aliquotes of 500 μl (equivalent to 100 μg of antigen) was administered intradermally at multiple sites on the rabbit after cleaning the immunization area with 70 % ethanol. Booster immunization was administered 4 weeks later with antigen emulsified in Freund's incomplete adjuvant (1:1).

2.8.2 Bleeding

Blood was collected in a clean glass tube washed with 0.9% normal saline from the lateral ear veins by making a gentle diagonal incision 6 days after the first booster. Repeated bleeding was done after 4^{th}-5^{th} day after every booster immunization thereon.

2.8.3 Separation of serum

The tube containing blood was allowed to stand tilted for clotting without any disturbance for usually 8-10 h at room temperature (RT). Serum was collected gently by a micropipette in a sterile tube. It was then centrifuged at 10,000 x *g* for 10 min at 4°C to remove any remaining traces of debris. The serum, containing polyclonal antibody, was concentrated by precipitating the immunoglobulin proteins with saturated ammonium sulfate.

2.8.4 Isolation of immunoglobins from serum proteins

Isolation of the IgG fraction in the complex mixture of serum proteins was carried out following method described by Harlow *et al.* (1988).

2.8.4.1 Required solutions

i) Ammonium sulfate saturated solution:

800 g l^{-1} in distilled water, pH 7, pre-cooled to 4 °C and stored in presence of ammonium crystals

ii) Dialysis buffer:

100 mM Tris-Cl buffer, pH 8 (degassed and filtered)

2.8.4.2 Methodology

A known volume of the serum was transferred into a beaker on ice. All the following steps were performed on ice. An equal volume of the saturated ammonium sulfate solution was added drop by drop while gently stirring the serum. Precipitation was allowed for around 1 h with constant but mild stirring. The solution was finally centrifuged at 10,000 x *g* for 10 min at 4 °C. The pellet was resuspended in cold saturated ammonium sulfate solution (5% of the original volume). Finally the resulting solution was dialyzed against three changes of the dialysis buffer over 48 h. The dialyzed solution was centrifuged at 10,000 x *g* for 10 min at 4 °C and the supernatant containing IgG fraction of polyclonal antibody was recovered and frozen. It was lyophilized and stored in cold until use.

2.9 OUCHTERLONY IMMUNODIFFUSION ASSAY

One % agarose (w/v) solution in normal saline (0.9 % NaCl with 0.02 % sodium azide) was prepared by heating it to about 90 °C with constant stirring. Approximately 2 ml of the agarose solution each was poured on a number of clean glass slides placed on a horizontal surface and was allowed to solidify at room temperature. Three gelation wells were punched 1 cm apart on each slide. The antibody was loaded in the central well while the antigen and BSA were loaded in the outer wells. Several such slides were loaded with serially diluted solutions of the same antigen and antibody. The gel slides were kept in a humid chamber maintained at 37°C for about 36 h. After the appearance of precipitin line, the gel was stained with coomassie brilliant blue. After destaining, the gel was photographed.

2.10 SNAKE VENOM PHOSPHODIESTERASE DIGESTION

The enzyme, snake venom phosphodiesterase (SVP) specifically attacks ADP-ribose polymers through direct interaction and endonuleolytic cleave of the pyrophosphate bonds. The enzyme action is reported to be very specific for poly-ADP-ribose (Sugimura, 1973).

2.10.1 Methodology

Histone proteins, isolated from spleen cells, were used at a concentration of 2 µg per 100 µl. The sample (500 µl) was mixed with 50 µl of freshly prepared SVP (4 mg ml^{-1}) and incubated at 37° C in a water bath for 15 min to allow degradation of polymer of ADP-ribose attached to histone proteins. After this, 100 µl of the samples were loaded on to a nitrocellulose membrane using a slot blotting apparatus under mild vacuum. The slot-blotted membrane was processed for immunodetection of poly-ADP-ribosylated proteins (see Sction 2.14.4) as well as total protein staining using ink (see Section 2.14.4). SVP untreated histone sample was used as control and double distilled water as a blank sample. The pixel intensities of the bands were quantified and analyzed by an imaging densitometer (Bio-Rad GS-690 Imaging Densitometer/ Molecular Analyst PC software).

2.11 SAMPLE PREPARATION

Liver, spleen, and femur bone were taken out from the untreated (control), treated and tumor bearing mice. The animals were sacrificed by cervical dislocation. Liver homogenate (usually 0.15 g in 1.5 ml), spleen-cell homogenate (all cells recovered from one spleen in 1 ml) and bone marrow-cell homogenate (all cells from two femurs in 0.5 ml) were prepared using a motorized tissue homogenizer in ice cold PBS, pH 7.4. Supernatants of all the homogenates after centrifugation at 10,000 x *g* for 15 min were used for assay of cellular poly-ADP-ribosylation (PAR) (see Section 2.13). In addition, histone proteins isolated from 60 x 10^6 spleen cells as well as ascitic cells (see Section 2.12) were also employed for PAR assay.

2.12 ISOLATION OF HISTONE PROTEINS

The method established in the laboratory for isolating histone proteins was followed with some alterations (Saikia, 1996).

2.12.1 Required Solutions

SPC buffer:

Potassium phosphate buffer, pH 6.8	1 mM
Sucrose	0.32 M
$CaCl_2$	1 mM
DTT	1 M

2.12.2 Methodology

Histone proteins were isolated from spleen of both normal (control) and treated animals. Spleen was excised and cells were squashed out in 1 ml of cold PBS. Cell suspension was prepared by repeated mild agitation with a pipette. In a volume of 50 µl of the buffer containing 60 x 10^6 cells, 0.5 ml of SPC buffer, 20 µl bench HCl (36.46 % conc. HCl) and 12 µl of ß- mercaptoethanol were added and mixed thoroughly by cyclomixer. The mixer was incubated on ice for 60 min and centrifuged at 10,000 x *g* for 5 min in a microcentrifuge. To the supernatant, 0.75 g ml^{-1} urea was added and vortexed till it dissolved properly. Finally, 5 µl of 0.2 % phenolphthalein and 0.05 x volume of 1 M DTT were added. A further addition of 50 µl bench ammonia gave a characteristic pink colour indicating the completion of the isolation. After 5 min, glacial acetic acid was added to the

isolate to a final concentration of 1 M. The content of histone proteins was estimated by the method of Bradford (see Section 2.19).

2.13 SLOT BLOTTING

Samples for slot blot assay were heat inactivated by boiling in a water bath for 5 min in order to inactivate endogenous phosphatase enzyme activity (Sharan *et al.*, 1998b). Samples were appropriately diluted with double distilled sterile water to get final concentration of 2 µg proteins per 100 µl volume loaded onto each slot. Prior to loading the samples, the nitrocellulose (NC) membrane was activated by immerging in double distilled water in a gel tray. It was kept for 15 min. The samples were then slot blotted onto NC membrane using Bio-dot sf® microfiltration apparatus (Bio-Rad) connected to a vacuum pump. The process lasted about 20 min.

2.14 WESTERN BLOTTING

The samples to be Western blooted were first subjected to SDS-PAGE. The gel was then electroblotted on nitrocellulose membrane to get the Western blot.

2.14.1 SDS-polyacrylamide gel electrophoresis

The method described by Laemmli (1970) was followed with minor modifications.

2.14.1.1 Requirements for gel preparation

i) Stock solutions: The following five stock solutions were prepared:

Acrylamide solution: Acrylamide Bis-acrylamide (filtered through Whatmann paper)	30 % 0.8 %;
Tris-Cl buffer, pH 8.8	1 M
Tris-Cl buffer, pH 6.8	1 M
SDS solution	10 %
Ammonium per sulfate (freshly prepared)	10 %

TEMED was directly used from the bottle.

ii) Electrophoresis buffer: It consisted of the following components:

Tris-Cl buffer, pH 8.8	25 mM

Glycine	192 mM
SDS	0.1 %

iii) Sample buffer (5 x): The composition of the buffer was:

Tris-Cl buffer, pH 6.8	0.5 M
Sucrose	5 g
SDS	0.5 g
ß-mercaptoethanol	0.25 ml
Bromophenol blue (0.5 % w/v solution in water)	1 ml
Double distilled water to final vol.	10 ml

The buffer concentrate was stored frozen in small aliquots.

iv) Separating gel

For preparing 10 ml of separating gel solution of 12 % acrylamide concentration, the following stock solutions were mixed together:

Acrylamide solution	4 ml
1 M Tris-Cl buffer, pH 8.8	3.73 ml
10 % SDS	100 µl
Double distilled water	2.10 ml

After degassing the mixture for 15 min, 30 µl each of TEMED and freshly prepared 10 % ammonium per sulfate were added. The solution was poured into pre-assembled casting glass plate sandwiches and was allowed to polymerize after overlaying with water. Polymerization usually took about 20 min at RT.

iv) Stacking gel

Similarly, for 5 ml of 3 % stacking gel solution, the following stock solutions were mixed together:

Acrylamide solution	500 µl
1 M Tris-Cl buffer, pH 6.8	625 µl
10 % SDS	50 µl
Double distilled water	3.8 ml
TEMED	12 µl
10 % APS	12 µl

After removing the water overlay from the polymerized separating gel, comb was fixed and stacking gel solution was poured. It was allowed to polymerize. The polymerization usually took about 30 min at RT.

2.14.1.2 Electrophoresis

The samples mixed with 1 x sample buffer were kept in boiling water bath for 3 min. About 12-15 µl of the samples were loaded in each well of the gel. The electrophoresis was carried out at a constant voltage of 25 V cm^{-1} for 1 h in a Mini-PROTEAN II electrophoresis cell.

2.14.1.3 Electroblotting

The following buffer was prepared:

Towbin buffer:

Tris-Cl buffer, pH 8.3	25 mM
Glycine	192 mM
Methanol	20 %

The samples separated on SDS-PAGE gel were electrotransferred onto activated NC membrane (in Towbin buffer for 15 min) in a mini Trans-blot electrophoretic transfer cell. The electroblotting was carried out in chilled Towbin buffer at a constant voltage of 100 V for 1 h.

2.15 IMMUNODETECTION OF POLY-ADP-RIBOSYLATED PROTEINS

Standard immunoprobing protocol was employed with slight modifications (Sharan *et al.*, 1998b). The working solutions and their compositions for the assay are shown below:

i) Tris buffered saline (TBS):

Tris-Cl buffer, pH 7.5	20 mM
NaCl	500 mM

ii) Tween-tris buffered saline (TTBS):

Tris-Cl buffer, pH 7.5	20 mM
NaCl	500 mM
Tween-20	0.05 %

iii) Blocking solution (BS):

Non-fat dry milk	5 % in TBS

iv) First Ab solution:

Polyclonal rabbit IgG	1: 500 in BS

v) Second Ab solution:

Anti-rabbit IgG ~ Alkaline phosphatase conjugate	1:15,000 in BS

vi) BCIP/NBT solution:

Each tablet of BCIP/NBT	Dissolved in 10 ml of double distilled water

2.15.1 Methodology

All incubations and reaction steps were maintained at 37 °C with gentle intermittent agitation unless otherwise mentioned. The sequenctial steps of the method, as performed in the assay, are described on the next page:

Slot or Western-blotted NC membrane saturated in 15 ml of BS for 45 min with gentle shaking at RT .

↓

The BS was decanted and the NC membrane washed twice, each for 5 min, in 10 ml of TTBS at RT.

↓

The NC membrane was then incubated in 15 ml of first-AB (polyclonal Ab against ADP-ribose polymer) for 45 min.

↓

The NC membrane was washed with 10 ml of TTBS for 5 min at RT

↓

The NC membrane was now incubated in 15 ml of second-Ab~alkaline phosphate conjugate solution for 45 min.

↓

The NC membrane was washed with TTBS for 10 min with occasional shaking at RT.

↓

The NC membrane was washed with TBS for 10 min with occasional shaking at RT.

↓

The NC membrane was then transferred in BCIP/NBT substrate solution and incubated optimum intensity of color was developed (7 to 15 min).

↓

The NC membrane was washed twice in glass double distilled water for 5 min each at RT.

↓

The NC membrane was then air dried and the pixel intensities of the coloured bands were quantified and analyzed by a GS 690 densitometer and Kodak Digital Camera using Molecular Analyst PC software 1. 5 and KDS 1D respectively.

2.16 TOTAL PROTEIN STAINING ON NITROCELLULOSE MEMBRANES

A replicum of the slot or western blotted NC membrane was stained for 3-4 h in 0.2 % Rotring ink prepared in PBS containing 0.3 % tween 20.

2.17 CHROMATIN ISOLATION

The method by Bellard *et al.* (1989) was followed with some additional steps for studying intact chromatin structure.

2.17.1 Reagents required

i) Phosphate buffered saline (PBS), pH 7.4

ii) TK buffer:

Tris-Cl buffer, pH 7.5	10 mM
$CaCl_2$	1 mM

2.17.2 Methodology

All steps were conducted on ice. Spleen was excised and cells were squashed out in a petridish containing chilled PBS, pH 7.4. Cell suspension was prepared by repeated mild agitation with pipette. The suspension was centrifuged at 1000 x *g* for 3 min and pellet was suspended in TK buffer. After incubating for 10 min on ice, cells were mechanically broken by mild homogenizing for 5 min. The homogenate was centrifuged at 1000 x *g* for 5 min and resuspended in TK buffer. After another step of centrifugation the nuclear pellet was washed successively in low ionic strength medium, i. e., 2 mM and 0.4 mM tris-Cl buffer, pH 8. The washed nuclear pellet was resuspended in ice cold double distilled H_20 and the chromatin was allowed to swell overnight at 4°C. The mixture was gently stirred on the following day. This solution has been taken as chromatin preparation taken as chromatin. Diphenylamine test was carried out to estimate the concentration of chromatin (See section 2.20).

2.18 DNase I DIGESTION AND AGAROSE GEL ELECTROPHORESIS

2.18.1 Reagents required

i) Digestion buffer:

KCl	85 mM

$CaCl_2$	1 mM
HEPES	5 mM
Sucrose	5%

ii) DNase I stock buffer:

Tris-Cl buffer, pH 7.5	20 mM
NaCl	50 mM
Glycerol	50 %
DTT	1 mM
BSA	100 µg/ ml

DNase I was dissolved in this stock buffer at a concentration of 1 mg/ ml (equivalent to 2300 units), aliquoted and stored at -20 °C.

iii) Stop buffer:

Tris-Cl buffer, pH 7.5	50 mM
NaCl	150 mM
EDTA	15 mM
SDS	0.3 %

iv) Electrophoresis buffer (TAE buffer):

Tris acetate, pH 8.42	40 mM
EDTA	MM

v) Sample buffer:

Sucrose	40 %
Bromophenol blue	0.25 %

2.18.2 Methodology

DNase I digestion of the chromatin was carried out for 2 min at 37°C. The digestion cocktail consisted of the following:

Chromatin preparation	12.5 µl (100 µg DNA)
Digestion buffer	9. 075 µl
DNase I	4.35 µl (10 U)

Adding 4.35 µl of stop buffer terminated the digestion reaction.

2.18.3 Agarose gel electrophoresis

The standard protocol for agarose gel electrophoresis was followed (Asubel *et al.*, 1995)

After diluting the chromatin cocktail with $1/6^{th}$ volume of sample buffer, the mixtures (5 μg DNA/ well) were analyzed by 0.6 % agarose gel electrophoresis using submarine gel electrophoresis apparatus GNA-100 at 10 V cm^{-1} for 60 min using TAE buffer. The gel, stained in 0.7-μg ml^{-1} ethidium bromide, was photographed on a mini transilluminator using DC120 digital zoom camera attached to Zenith PC LR4Dc.

2.19 ESTIMATION OF PROTEIN

The amount of proteins in all samples was estimated following method of Bradford (1976) using BSA as a standard.

2.19.1 Preparation of stock and working solutions

The stock solution was prepared by dissolving 100 mg of Coomassi brilliant blue G-250 in 50 ml of 95 % ethanol. To this added 85 % w/v phosphoric acid, mixed thoroughly and stored refrigerated. The working solution was prepared by mixing 15 ml of stock solution in 85 ml of double distilled water. It was filtered through Whatman # 1 filter paper after properly mixing and the filtrate was used.

2.19.2 Methodology

A known volume of protein sample was taken and its volume was made up to 0.1 ml with distilled water. To this 5 ml of the working Bradford solution was added and vortexed gently. The absorbance of the solution was read at 595 nm after 5 min of incubation at RT.

2.20 ESTIMATION OF DNA

The content of DNA was determined following the method described by Burton (1968) with some alterations. Commercially available DNA (Sodium salt type III from Salmon testis) was used as a standard.

2.20.1 Preparation of diphenylamine reagent

1.5 g of diphenylamine was dissolved in 100 ml of glacial acetic acid and 1.5 ml of concentrated sulfuric acid. This reagent was stored at room temperature.

2.20.2 Methodology

Biological sample to be assayed was taken and its volume made up to 0.1 ml with distilled water. To this 2 ml of diphenylamine reagent was added and mixed thoroughly. The cocktail was incubated in a boiling water-bath for 10 min. The absorbance of the solution was measured at 595 nm after cooling down.

2.21 ESTIMATION OF RNA

The method of Merchant *et al.* (1969) was employed with some changes. Commercially available RNA (Type III from Bakers Yeast) was used as a standard.

2.21.1 Preparation of Orcinol reagent

One g of Orcinol was dissolved, immediately before use, in 100 ml of concentrated HCl containing 0.5 g of ferric chloride.

2.21.2 Methodology

Biological sample was taken and its volume was made up to 0.1 ml with distilled water. To this 2 ml of Orcinol reagent was added and mixed thoroughly. The cocktail was incubated in boiling water bath for 20 min. The absorbance of the solution was measured at 660 nm after cooling down to RT.

2.22 QUANTIFICATION AND STATISTICAL ANALYSIS

An electrophoresis documentation and analysis system, Kodak Digital Science 1D software, using a Kodak digital camera was used for quantification and analysis of the data. In some cases Densitometer GS 690 using Bio-Rad 1D software (Molecular Analyst PC software, version 1.5) was employed for the analysis. Mean intensity or pixel density of the band was used for monitoring PAR level of total cellular proteins as well as histone proteins. Statistical calculations and plotting of graphs were done with the help of Origin (version 3.5) and Excel programs, respectively. All values were expressed as mean ± SEM. $P < 0.05$ was considered statistically significant.

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CHAPTER III

RESULTS

3.1 IMMUNOGENICITY OF THE ANTISERUM

The immunogenicity of the antiserum against the antigen was ascertained by Ouchterlony immunodiffusion assay. Fig. 5 depicts qualitative precipitin line formation between soluble ADP-ribose polymer antigen (Ag) and the polyclonal antibody (Ab) of the serum but not between the Ab and BSA, a test protein. Precipitin line (arrow) was formed in the equivalence zone confirming the immunogenicity between the antiserum and poly-ADP ribose.

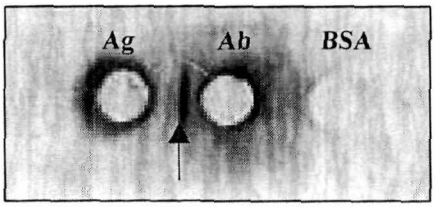

Figure 5: Formation of precipitin line (arrow) between ADP-ribose polymer Ag and polyclonal Ab raised against it in serum in 1% agarose gel by Ouchterlony immunodiffusion assay. Antiserum containing Ab was placed in the central well while Ag (ADP-ribose polymer isolated from normal mouse spleen cells) in the left and BSA in the right well, respectively. Diffusion occurred at 37°C in a moist chamber for about 36 h.

3.2 SPECIFICITY OF THE POLYCLONAL ANTIBODY

The specificity of the antibody-antigen interaction was ascertained by snake venom phosphodiesterase (SVP) induced specific degradation of the ADP-ribose polymer (Sugimura, 1973) from histone proteins isolated from normal mouse spleen cells.

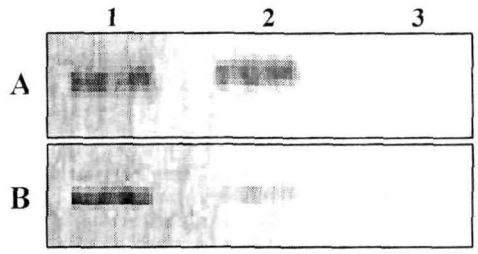

Figure 6: Specificity of immune-interaction between anti poly-ADP-ribose polyclonal antibody and poly-ADP ribose antigen. The ADP-ribose polymers associated with histones isolated from normal mouse spleen was selectively degraded for 15 min at 37°C by SVP enzyme before slot blotting and immunodetection. Row A: ink stain, row B: immunodetection, lane 1: non-degraded control sample, lane 2: 15 min SVP treated sample and lane 3: blank sample (double distilled water).

Fig. 6 shows ink stained (A) and immunodetected (B) histone proteins immobilized on nitrocellulose membrane following slot blotting. Lane 1 in row B showed high intensity signal following color development in immunodetection indicating presence of poly-ADP-ribose on histones in the control sample without SVP treatment. On the contrary, in the

histone sample treated with SVP for 15 min at 37°C, there was no detectable signal after immunodetection (lane 2 in row B). The signal was as negligible as in lane 3, which had double distilled water.

3.3 EFFECTS OF DIMETHYLNITROSAMINE ON POLY-ADP-RIBOSYLATION OF TOTAL CELLULAR PROTEINS

Levels of PAR were monitored in liver, spleen and bone marrow of untreated and DMN treated animals. Fig. 7 shows the ink stained (lane 1) and immunodetected (lane 2) immobilized proteins on nitrocellulose membranes from all the samples used for analysis, i.e., histone proteins isolated from spleen cells, liver homogenate, spleen cells homogenate and BMC homogenate. Lane 1 in all the panels represent the total amount of proteins loaded on the membrane while lane 2 shows extent of PAR of the loaded corresponding proteins. The color intensities developed on slot blotted bands after immunodetection were higher in homogenates of all tissues (lane 2 of panels B, C and D) than that of isolated histone proteins from spleen cells (lane 2 of panel A).

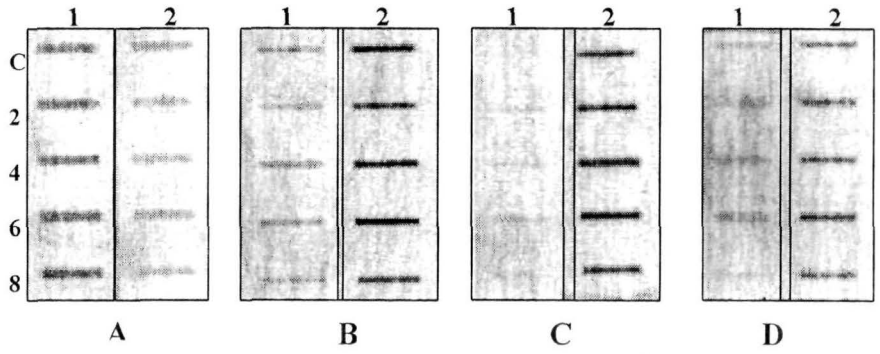

Figure 7: Slot blotted nitrocellulose membranes stained with ink (lane 1) and immunodetected (lane 2) showing proteins from various tissues of untreated and diethylnitrosamine (DMN) treated animals. Panel A - isolated histones from spleen cells, panel B - liver homogenate; panel C - spleen cell homogenate and panel D - BMC homogenate; blots from top to bottom: normal control (C) and 2, 4, 6 & 8 weeks DMN treated, respectively.

3.4 EFFECTS OF 3-AMINOBENZAMIDE ON POLY-ADP-RIBOSYLATION OF TOTAL CELLULAR PROTEINS

Levels of PAR were studied following 3-aminobenzamide (3-AB) treatment for a period up to 8 weeks. Fig. 8 depicts the visual impression of the color intensities of slot blotted samples on nitrocellulose membranes developed after ink staining (lanes 1) and after immunodetection (lanes 2) in all panels. While the colour signals given by ink staining of

total cellular proteins slot blotted on the membranes (lanes 1) were more intensed, the immunodetected slots (lanes 2) showed less intensed signals. The extent of PAR was lowered in all the tissues after exposure to 3-AB as compared to their controls as revealed by immunodetected slot blots (lane 2).

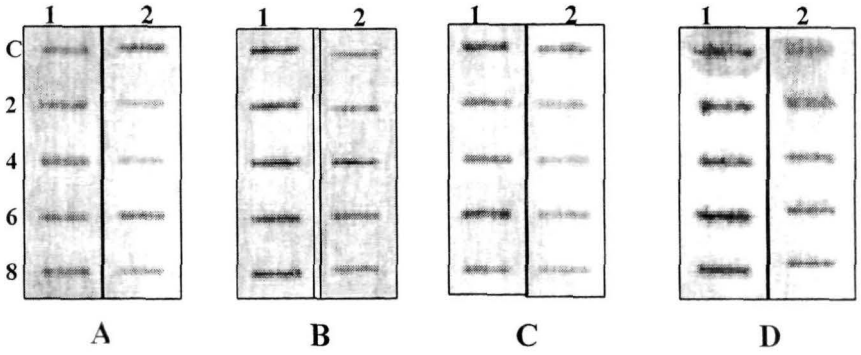

Figure 8: Slot blotted nitrocellulose membranes stained with ink (lane 1) and immunodetected (lane 2) showing proteins from various tissues of untreated and 3-aminobenzamide (3-AB) exposed animals. Panel A - isolated histones from spleen cells, panel B - liver homogenate; panel C - spleen cell homogenate and panel D - BMC homogenate; blots from top to bottom: normal control (C) and 2, 4, 6 & 8 weeks 3-AB treated, respectively.

3.5 EFFECTS OF DIMETHYLNITROSAMINE + 3-AMINOBENZAMIDE ON POLY-ADP-RIBOSYLATION OF TOTAL CELLULAR PROTEINS

Levels of total PAR of cellular proteins were also monitored in mice tissues after simultaneous exposure of the mice to chronic dose of DMN + 3-AB. Fig. 9 shows the visual observation of the extent of PAR after combined regime of DMN + 3-AB. The PAR of total histone proteins from spleen cells was much inhibited (lane 2 of panel A) as compared to total cellular proteins of all the tissues investigated (lanes 2 of panels B, C and D).

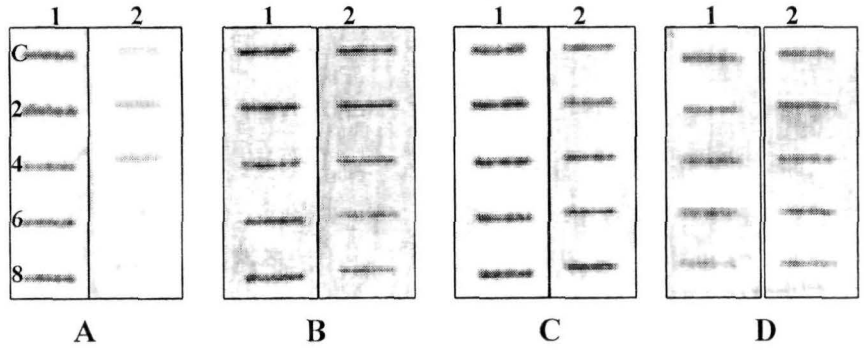

Figure 9: Slot blotted nitrocellulose membranes stained with ink (lane 1) and immunodetected (lane 2) showing proteins from various tissues of untreated mice and those exposed to dimethylnitrosamine (DMN) in conjunction with 3-aminobenzamide (3-AB). Panel A - isolated histones from spleen cells, panel B - liver homogenate; panel C - spleen cell homogenate and panel D - BMC homogenate; blots from top to bottom: normal control (C) and 2, 4, 6 & 8 weeks DMN + 3-AB treated, respectively.

3.6 QUANTITATIVE ANALYSIS OF SLOT BLOTS

Level of PAR of total cellular proteins of liver, spleen cells and BMC and total histone proteins of spleen cells at biweekly period were quantified following exposure to DMN, 3-AB and DMN + 3-AB. Slot blotted bands developed after immunodetection using polyclonal antibody against ADP-ribose polymer, were quantified by KDS 1D software from independent experimental sets of all the three treatment groups. Mean intensity of the bands was used as quantifying parameter. Fig. 10 shows the levels of PAR of total cellular proteins in liver, spleen cells and bone marrow cells and total histone proteins from spleen cell histones from all treatment groups examined. The graphical representations were plotted using the mean value of different treatment groups. In liver, the target organ for DMN induced carcinogenesis, there was non-significant slight enhancement of the level up to 4 weeks followed by significant reduction in the later part of treatment. In spleen cells and bone marrow cells, the level of PAR in total cellular proteins generally went down during the exposure period. The isolated histone proteins from spleen cells also showed similar trend. The siginificant reduction in the level of PAR was observed in 6^{th} week in total cellular proteins of liver, spleen cells and bone marrow cells as well as histones of spleen cells. Under the combined treatment of DMN + 3-AB, the decreased in the level of PAR was potentiated in most cases. The lowering of PAR level in all tissues was significant in 4 weeks or later in most tissues except for bone marrow cells. The influence of 3-AB, inhibitor of PARP also showed that the levels of PAR of total cellular proteins in all tissues and histone proteins from spleen cells were progressively inhibited as expected. In this case also, significant inhibition was noticed.

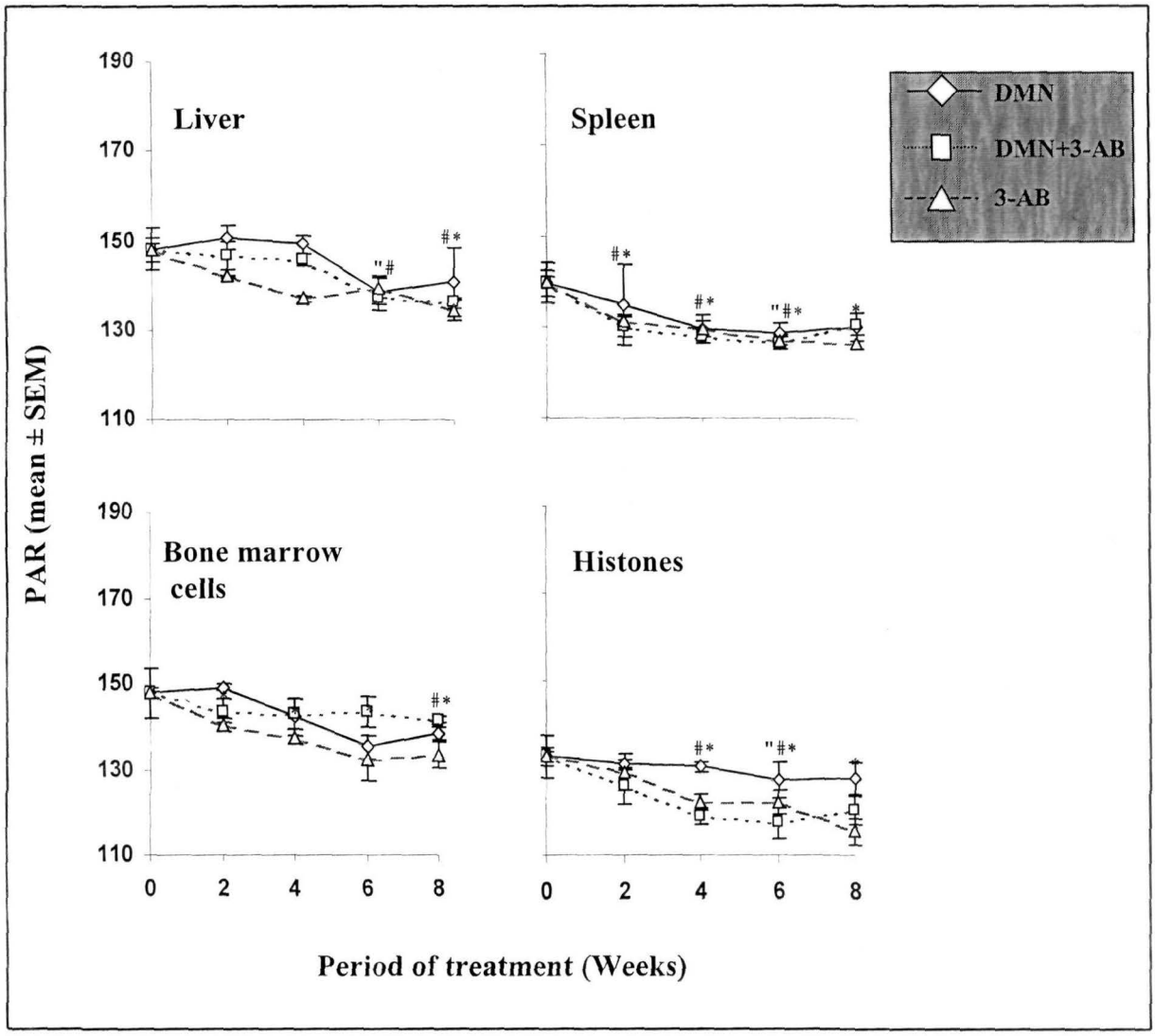

Figure 10: Levels of PAR of total cellular proteins of different tissues from unexposed control mice and those under different treatment conditions of dimethylnitrosamine (DMN), 3-aminobenzamide (3-AB) and DMN + 3-AB for a period up to 8 weeks. ": siginificant decrease in level of PAR after DMN treatment; #: significant decrease after DMN + 3-AB treatment; *: significant decrease after 3-AB treatment. Data obtained from three independent experimental sets each with two replicates of slot blots.

3.7 SDS-PAGE PROFILES OF ISOLATED AND STANDARD MARKER HISTONES

Fig. 11 shows comparative electrophoretic profiles of histones isolated from normal mouse spleen cells and commercially available standard histones (used as markers) from calf thymus. Rf value (distance moved by the protein to that moved by the dye front) of each histone in both gels was calculated using 1D Kodak digital camera and analytical software, KDS 1D. Rf values of different proteins were almost equal and this was employed for identification of different histone protein bands in this study, especially after western blotting. In this investigation, the protein bands representing H2b + H3 were analysed together the bands were overlapping to each other.

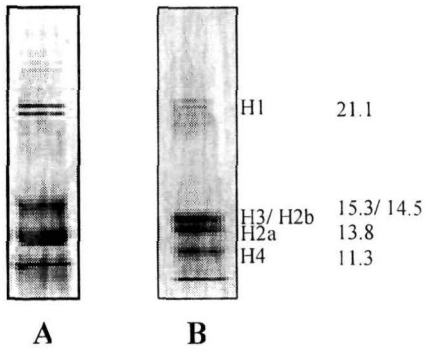

Figure 11: A comparison of the electrophoretic profiles of histone proteins isolated from normal mouse spleen cells (A) and the standard histones marker of calf thymus, Boeringer (B). The molecular weights of standard histone proteins in kDa are shown on the right.

3.8 SDS-PAGE PROFILES OF PROTEINS FOLLOWING DIMETHYL-NITROSAMINE TREATMENT

Fig. 12 shows SDS-PAG electropherogram of histones isolated from spleen cells, and homogenates of spleen cells and liver under the chronic treatment of chemical carcinogen, dimethylnitrosamine for a period up to 8 weeks. The electropherograms of proteins in all the cases indicated that between the normal and treated animals, there were no perceptible changes in the protein patterns. However, slight over- and under- expressions of proteins under the DMN influence were noticeable.

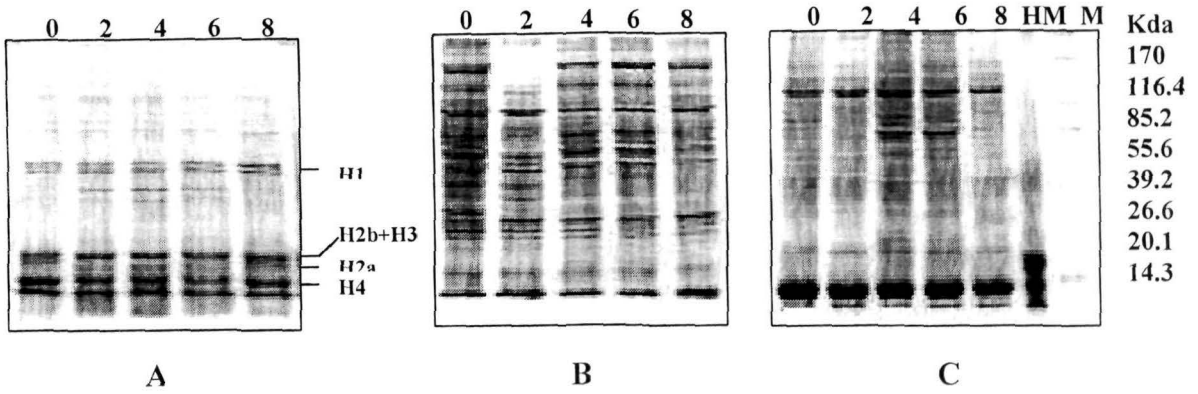

Figure 12: Sodium dodecyl sulphate-polyacrylamide gel electropherogram of different tissues from normal and chronically treated animals with DMN for a period up to eight weeks. Panel A - isolated histones from spleen cells; panel B - liver homogenate & panel C - spleen homogenate. Lanes left to right : 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively, lane HM and lane M represent standard histone markers (source - calf thymus) and calibration proteins (14.3-170 kDa) from Boeringer, respectively.

3.9 INK STAINED WESTERN BLOTS OF PROTEINS FROM SDS-PAGE AFTER DIMETHYLNITROSAMINE TREATMENT

The proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes in a transblot chamber.

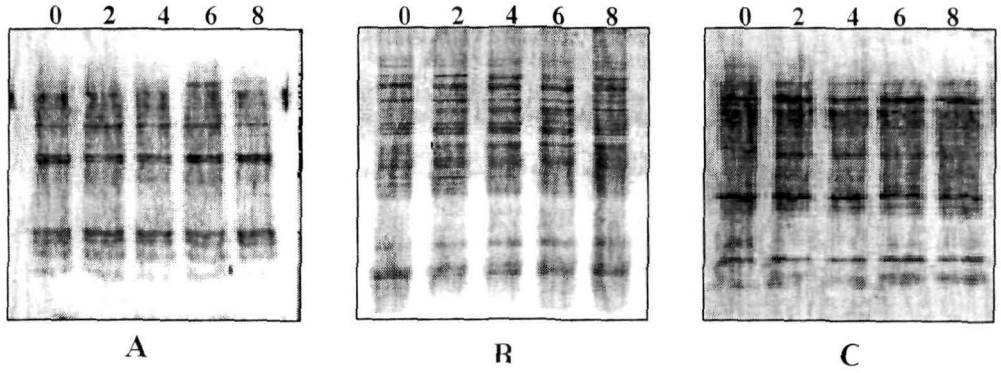

Figure 13: Ink stained western blots of SDS-PAG electropherogram of different tissues from normal and chronically treated animals with DMN for a period up to eight weeks. Panel A - isolated histones from spleen cells; panel B - liver homogenate & panel C - spleen cell homogenate. Lanes left to right: 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively.

Following the Western blotting the membranes were later processed for checking total protein content on nitrocellulose membranes by ink staining. They are the replicas of the gels and help in identification of individual proteins. Fig. 13 represents the ink stained Western blots of histones isolated from spleen (panel A), liver homogenate (panel B) and spleen cells homogenate (panel C).

3.10 IMMUNODETECTED PROTEINS ON THE WESTERN BLOTS FOLLOWING DIMETHYLNITROSAMINE TREATMENT

The level of PAR among individual proteins in cellular system under DMN treatment was studied. Following the Western blotting, the nitrocellulose membranes were processed for immunodetection assay using the polyclonal anti ADP-ribose polymer antibody. Fig. 14 shows PAR in isolated histones from spleen cells, liver and spleen cell homogenate.

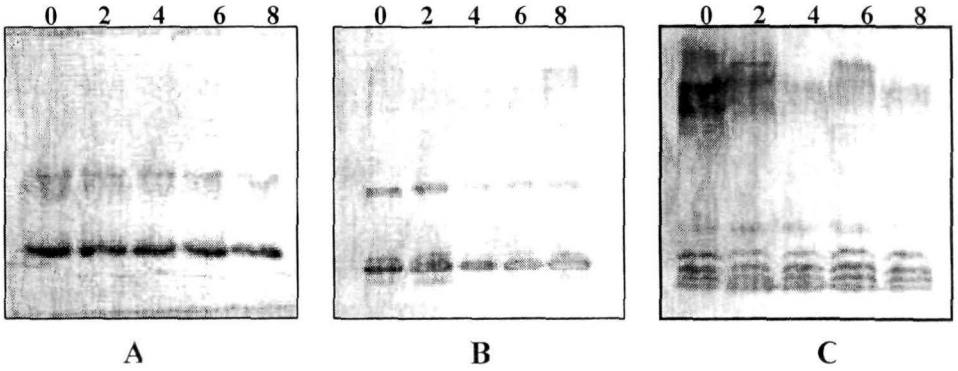

Figure 14: Immunoprobed western blot of different tissues from normal and chronically treated animals with DMN for a period up to eight weeks. Panel A - isolated histones from spleen cells; panel B - liver homogenate & panel C - spleen cell homogenate. Upper arrow - Immunodetected H1; lower arrow - Immunodwetcted H2b + H3. Lanes left to right: 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively.

The result shows that in the homogenates of liver (panel B) and spleen (panel C), primarily the histone proteins were poly-ADP-ribosylated. On the whole, PAR of histone proteins in the control samples appeared to be more ribosylated than DMN treated groups. Among the histone proteins, core histones were more poly-ADP-ribosylated than histone H1. In spleen cells homogenate, some high molecular weight non-histone proteins were also poly-ADP-ribosylated apart from histones. This was not observed in liver homogenate.

3.11 SDS-PAGE PROFILES OF PROTEINS FOLLOWING 3-AMINOBENZAMIDE TREATMENT

Similar experimental approach was carried out for 3-aminobenzamide treated mice. Fig. 15 depicts the SDS-PAGE profiles of histones isolated from spleen cells (panel A), homogenates of liver (panel B) and spleen cells (panel C). There were no distinguishable changes in the protein patterns of normal (control) and 3-AB treated groups of mice.

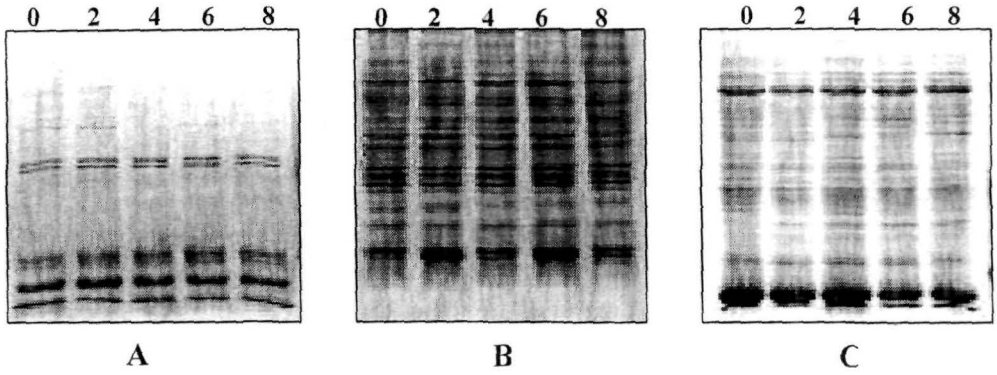

Figure 15: Sodium dodecyl sulphate-polyacrylamide gel electropherogram of different tissues from normal and chronically treated animals with 3-aminobenzamide for a period up to eight weeks. Panel A - isolated histones from spleen cells; panel B - liver homogenate & panel C - spleen cell homogenate. Lanes left to right : 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively.

3.12 INK STAINED WESTERN BLOTS OF PROTEINS FROM SDS-PAGE AFTER 3-AMINOBENZAMIDE TREATMENT

Fig. 16 shows ink stained western blotted total cellular proteins and histone proteins on nitrocellulose membrane from SDS-PAGE gel by electroblotting. Replicas of the original patterns of protein bands of the gels were obtained with good blotting efficiency.

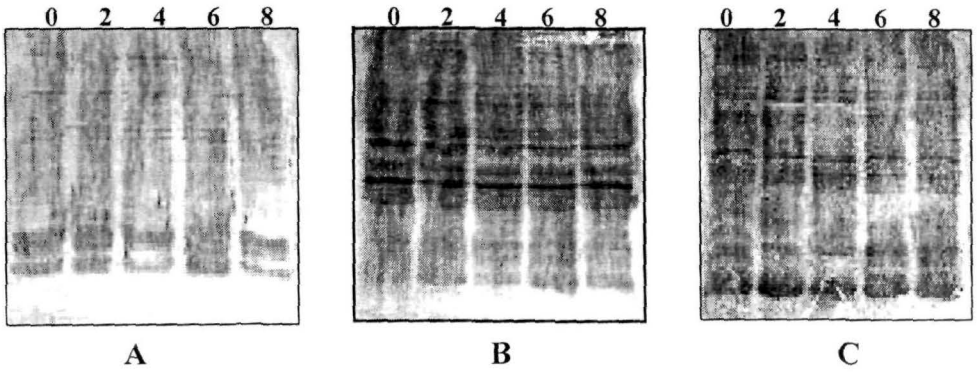

Figure 16: Ink stained Western blots of SDS-PAG electropherogram of different tissues from normal and chronically treated animals with 3-aminobenzamide for a period up to eight weeks. Panel A - isolated histones from spleen cells; panel B – liver homogenate & panel C – spleen cells homogenate. Lanes left to right: 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively.

3.13 IMMUNODETECTED PROTEINS ON THE WESTERN BLOTS FOLLOWING 3-AMINOBENZAMIDE TREATMENT

The immunoprobed Western blots (Fig. 17) show that even under the inhibitory action of 3-AB, poly-ADP-ribosylated proteins were detectable by immunoassay employed in this work in all the tissues examined. The histones, particularly the core histones, were the

main proteins that were poly-ADP-ribosylated. The distinction between control and 3-AB exposed groups, however, was less pronounced and nearly no high molecular weight proteins were poly-ADP-ribosylated.

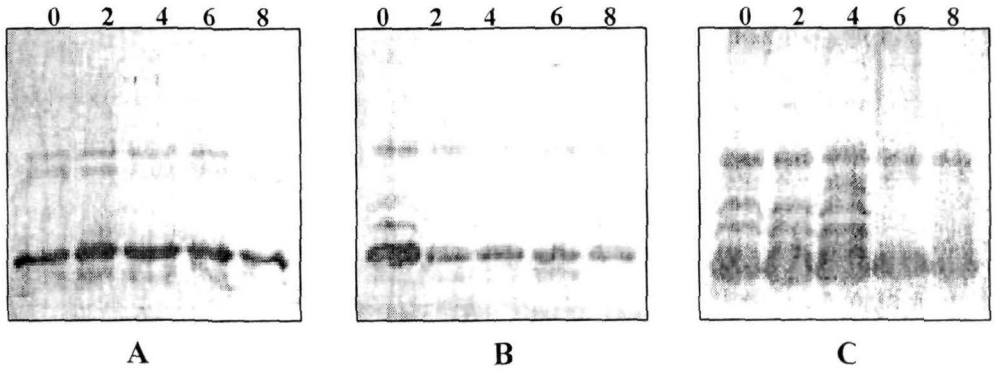

Figure 17: Immunoprobed Western blots of different tissues from normal and chronically treated animals with 3-AB for a period up to eight weeks. Panel A - isolated histones from spleen cells; panel B - liver homogenate & panel C - spleen cell homogenate. Lanes left to right: 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively.

3.14 SDS-PAGE PROFILES OF PROTEINS FOLLOWING COMBINED TREATMENT OF DIMETHYLNITROSAMINE + 3-AMINOBENZAMIDE

Proteins from different tissues of unexposed (control) and that of exposed mice to dimethylnitrosamine + 3-aminobenzamide were also separated by SDS-PAGE. Fig. 18 shows electropherogram of isolated histones from spleen cells (panel A), liver homogenate (panel B) and spleen cell homogenate (panel C). Even under the combined effect of DMN + 3-AB, the separating proteins for both control and treated animals showed little difference. There were no visible drastic changes in all tissues examined.

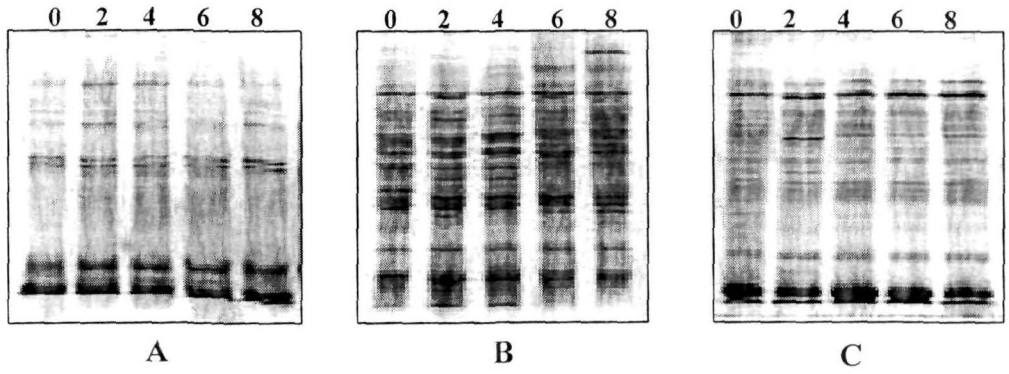

Figure 18: Sodium dodecyl sulfate-polyacrylamide gel electropherogram of different tissues from normal and treated animals with dimethylnitrosamine and 3-aminobenzamide in conjunction for a period up to eight weeks. Panel A - isolated histones from spleen cells; panel B - liver homogenate; C - spleen cells homogenate. Lanes left to right: 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively

3.15 INK STAINED WESTERN BLOTS OF PROTEINS FROM SDS-PAGE FOLLOWING DMN + 3-AB TREATMENT

The separated proteins after SDS-PAGE were transferred on to the nitrocellulose membrane (NC) by electrophoretic elution in a transblot chamber. The Western blotted proteins were stained with India ink for checking the presence of transferred proteins on to NC membrane. Fig. 19 shows the ink stained resolved proteins transferred on to NC membranes with good retention.

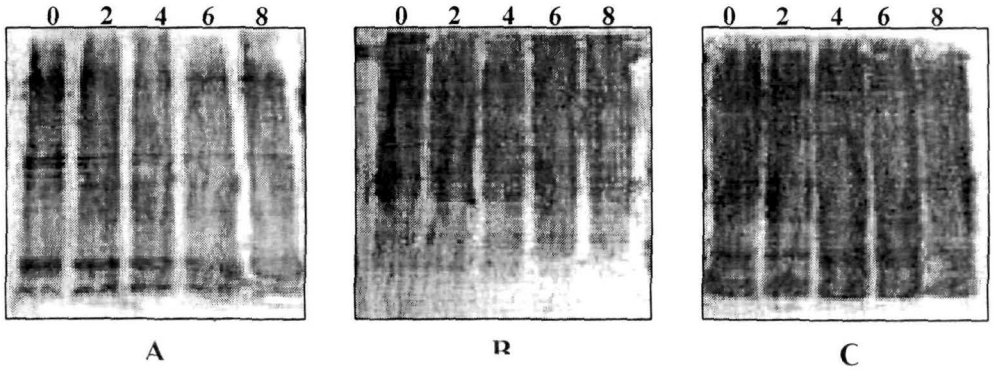

Figure19: Ink stained Western blots of SDS-PAG electropherogram of different tissues from normal and chronically treated animals with dimethylnitrosamine in conjunction with 3-aminobenzamide for a period up to eight weeks. Panel A - isolated histones from spleen cells; panel B - liver homogenate & panel C - spleen cells homogenate. Lanes left to right: 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively

3.16 IMMUNODETECTED PROTEINS ON WESTERN BLOTS FOLLOWING DIMETHYLNITROSAMINE + 3-AMINOBENZAMIDE TREATMENT

Fig. 20 shows immunoprobed Western blots following electrophoretic transfer of proteins on to nitrocellulose membrane under combined treatment of DMN + 3AB. The digitized pictographs of immunoblots show that PAR occurred mostly in histones among the total cellular proteins. In spleen cells homogenate, some proteins of high molecular weight as well as low molecular weight were ribosylated apart from the histones examined in this investigation. Progressive lowering of .PAR was evident in all cases after the combined treatment of DMN + 3-AB.

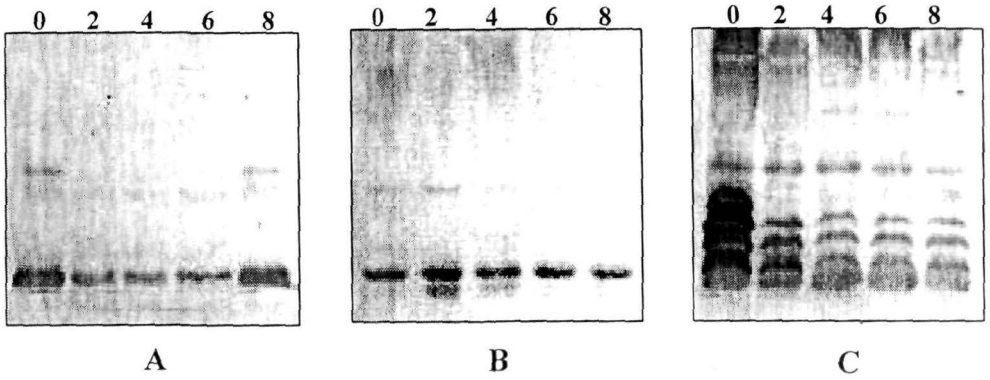

Figure 20: Immunoprobed western blot different tissues from normal and chronically treated animals with dimethylnitrosamine in conjunction with 3-aminobenzamide for a period up to eight weeks. Panel A- isolated histones from spleen cells; panel B - liver homogenate & C - spleen cell homogenate. Lanes left to right: 0 (control), 2, 4, 6 & 8 weeks of treatment, respectively.

3.17 QUANTITATIVE ANALYSIS OF WESTERN BLOT DATA

The status of PAR level in individual proteins of different tissues under the three treatment groups is better illustrated by quantification analysis. The results from the quantification data are shown by Fig. 21, 22, and 23 for liver, spleen cells and histones isolated from spleen cells respectively. Quantification of immunodetected proteins was done with the help of 1D Kodak digital camera attached with analytical software, KDS 1D. The results obtained from the analysis of these data are described below.

The graph depicts that extent of PAR of different histones of liver, spleen and histones of spleen were different. In the target tissue of DMN; liver, PAR response from different histones was significantly inhibitory in H1, H2b + H3 and H4 from 4^{th} week or later (Fig. 21). In spleen cells homogenate and isolated histones, the significant inhibitory effect was observed only in H1 in the 6^{th} and 8^{th} week of DMN exposure (Fig. 22, 23). However, the inhibitory consequence under DMN control appeared to be potentiated in simultaneous presence of 3-aminobenzamide (3-AB), the potent inhibitor of ADP-ribose polymer synthesis. PAR of H1 was significantly effected (Fig. 21). The related trend was observed in histones H2a, H2b + H3 and H4 particularly after 6 weeks of exposure (Fig. 21). Inhibitory response of PAR under 3-aminobenzamide separate treatment was pronounced in most cases (Fig. 21, 22, 23).

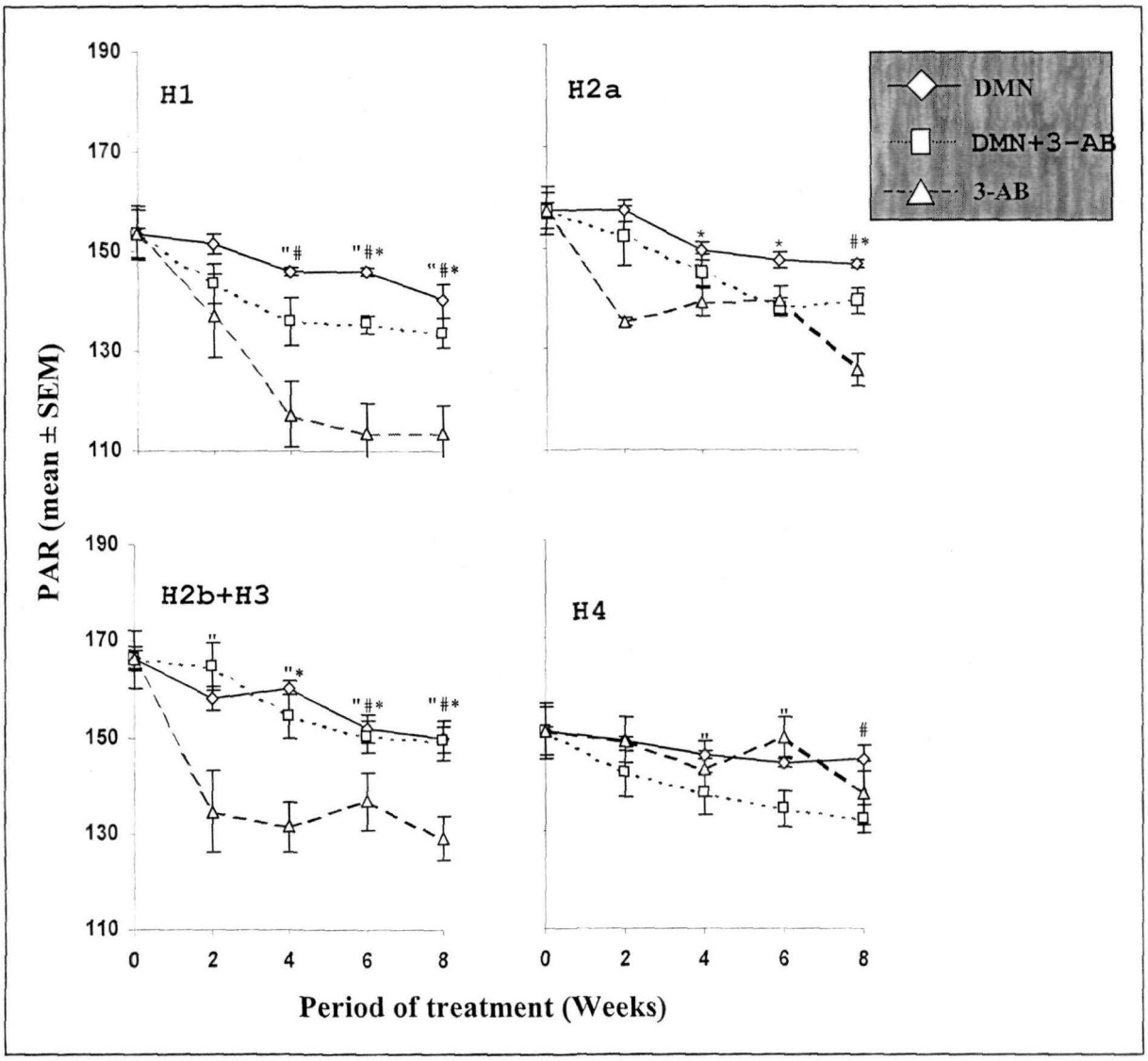

Figure 21: Levels of PAR of different histones of liver from unexposed control mice and those under different treatment conditions of dimethylnitrosamine (DMN), 3-aminobenzamide (3-AB) and DMN + 3-AB in combination for a period up to 8 weeks. ": siginificant decrease in level of PAR after DMN treatment; #: significant decrease after DMN + 3-AB treatment; *: significant decrease after 3-AB treatment. Data obtained from three independent experimental sets with two replicates of Western blots.

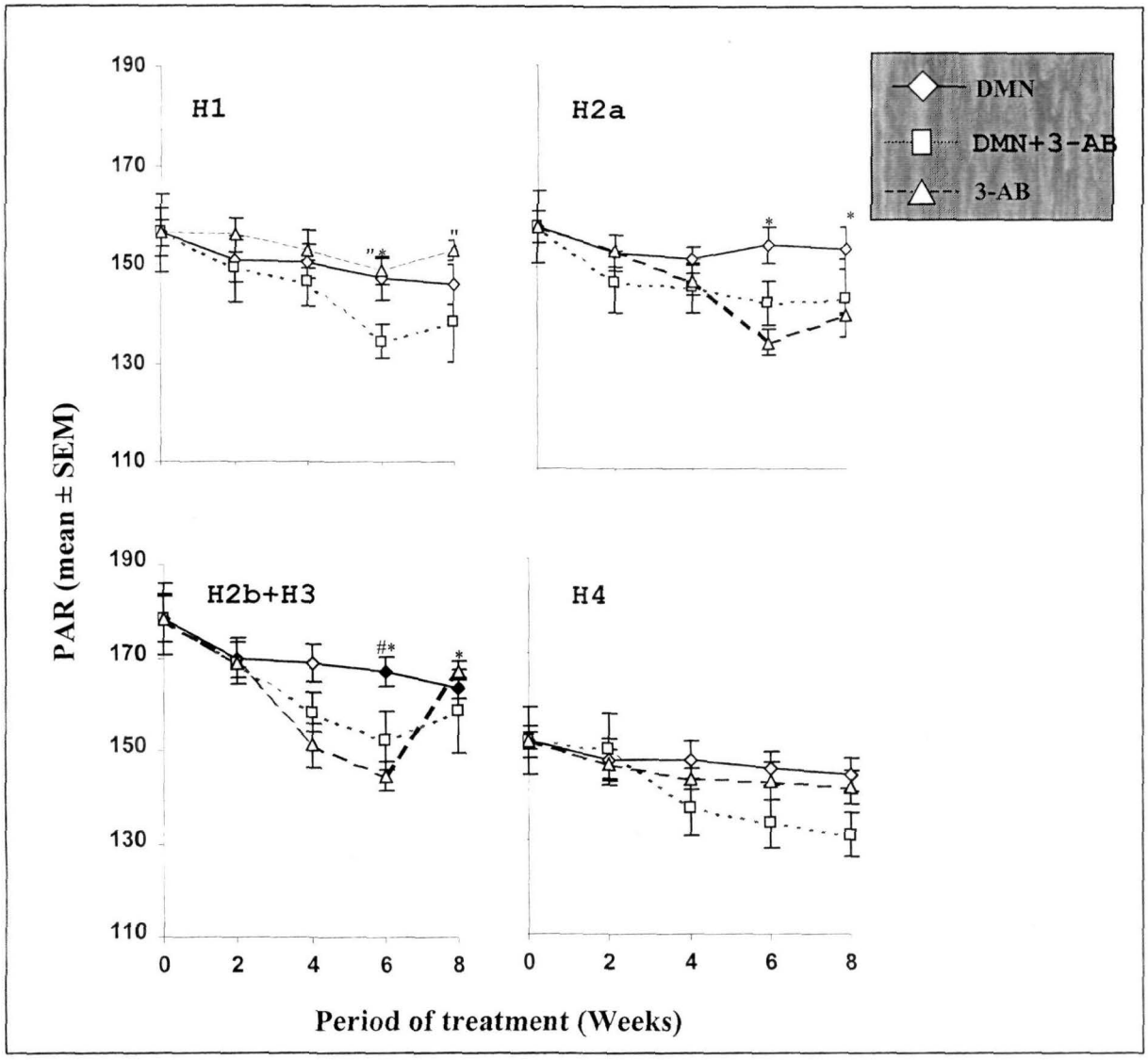

Figure 22: Levels of PAR of different isolated histones of spleen from unexposed control mice and those under different treatment conditions of dimethylnitrosamine (DMN), 3-aminobenzamide (3-AB) and DMN + 3-AB for a period up to 8 weeks. ": siginificant decrease in level of PAR after DMN treatment; #: significant decrease after DMN + 3-AB treatment; *: significant decrease after 3-AB treatment. Data obtained from three independent experimental sets with two replicates of Western blots.

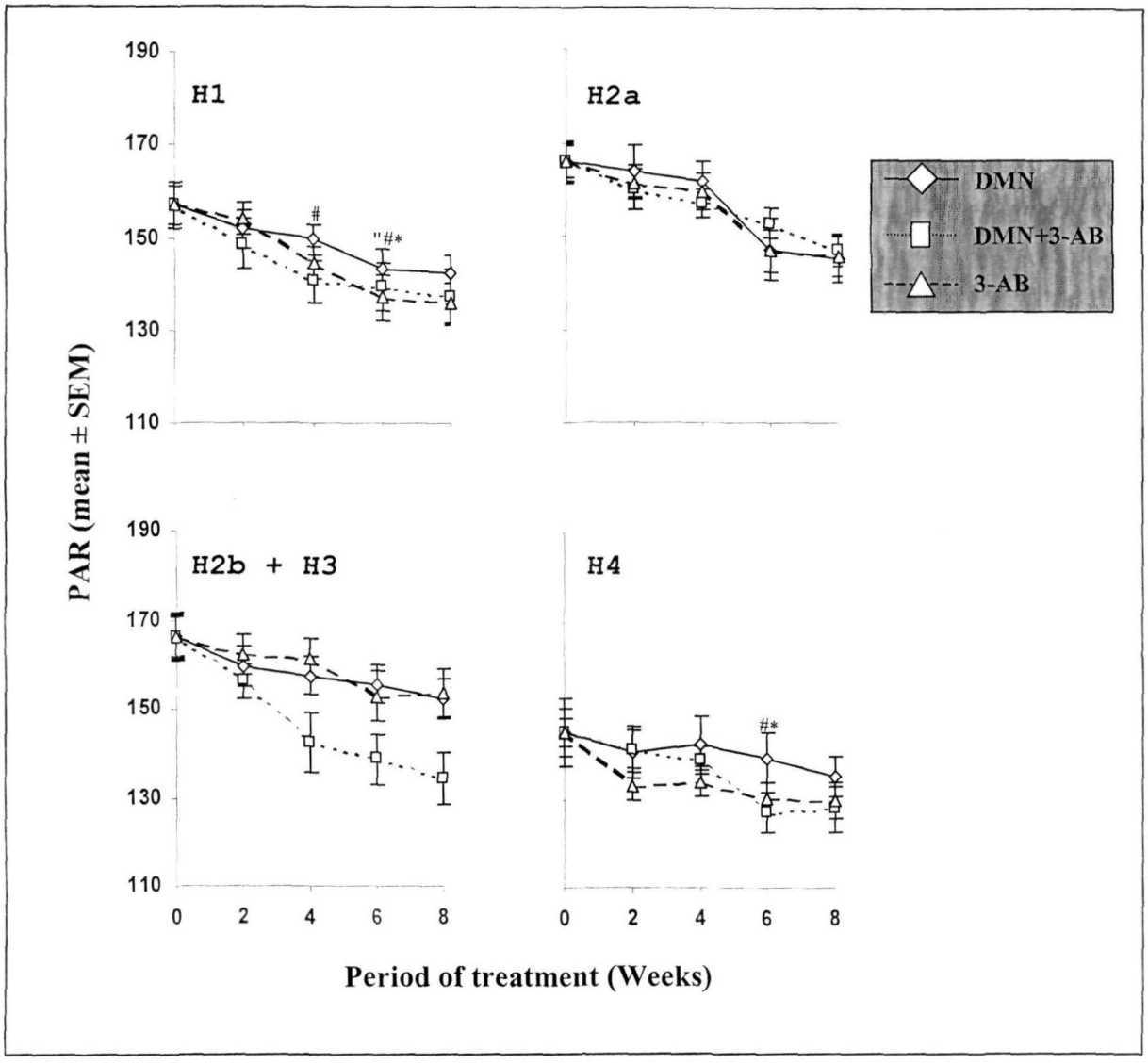

Figure 23: Levels of PAR of different histones of spleen homogenate from unexposed control mice and those under different treatment conditions of dimethylnitrosamine (DMN), 3-aminobenzamide (3-AB) and DMN + 3-AB for a period up to 8 weeks. ": siginificant decrease in level of PAR after DMN treatment; #: significant decrease after DMN + 3-AB treatment; *: significant decrease after 3-AB treatment. Data obtained from three independent experimental sets with two replicates of Western blots.

3.18 EFEECTS OF DMN, 3-AB AND DMN + 3-AB ON GENERAL PHYSIOLOGY

The DMN treated mice at a chronic dose of 10 mg kg^{-1} body weight became sluggish and lethargic during the treatment period. Similar was the case with group of mice exposed simultaneously to DMN and 3-AB. However, the mice animals under only 3-AB exposure showed normal activity level. On the whole there was no distinguishable sign of abnormality in any of the three groups of mice exposed to DMN, 3-AB or DMN + 3-AB throughout the period of treatment.

Fig. 24 depicts general physiological accounts before and after treatment of mice with dimethylnitrosamine. The body weights (panel A) of the treated mice remained invariant during the course of treatment as compared to the untreated control mice. Very slight increase in the body weights appeared to be related to normal growth of the mice. The number of cells in spleen (panel B) and femurs or bone marrow cells (panel C) of normal unexposed and chronically exposed mice to DMN also remained invariant during the course of this investigation.

Panel D panel E depicts total cellular protein content in liver (mg/g tissue) and spleen (mg/ million cells) of normal and chronically exposed mice to DMN at a dose rate of 10 mg. kg. $^{-1}$ b. wt for a period up to eight weeks. There were no striking changes in total protein content during the period of treatment although time dependent tendency of slight increase and decrease was noticed. In case of histone content (panel F) also, there was no distinct variation during the treatment period. Likewise the above parameters were checked for the other two treatment groups; 3-AB and also DMN + 3-AB. Similar results were obtained in most cases which are shown by Fig. 21 and 22 respectively.

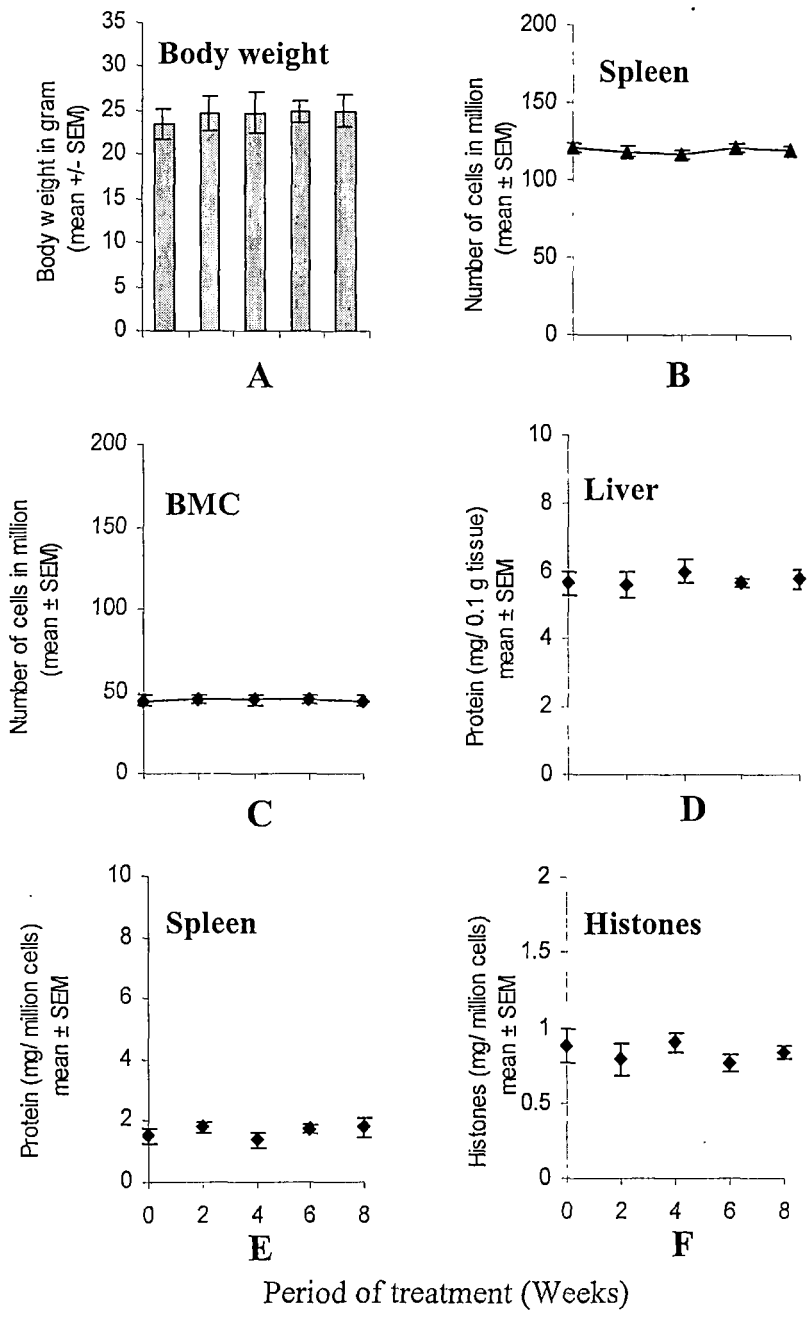

Figure 24: Effect of dimethylnitrosamine on general physiology of treated mice as compared to untreated (control) for a period of 8 weeks. Panel A: body weights; panel B: cell numbers in spleen; panel C: cell numbers in BMC; panel D: total protein contents in liver; panel E: total protein contents in spleen and panel F: histones content in spleen. Values represent mean of three independent experimental groups with two mice in each.

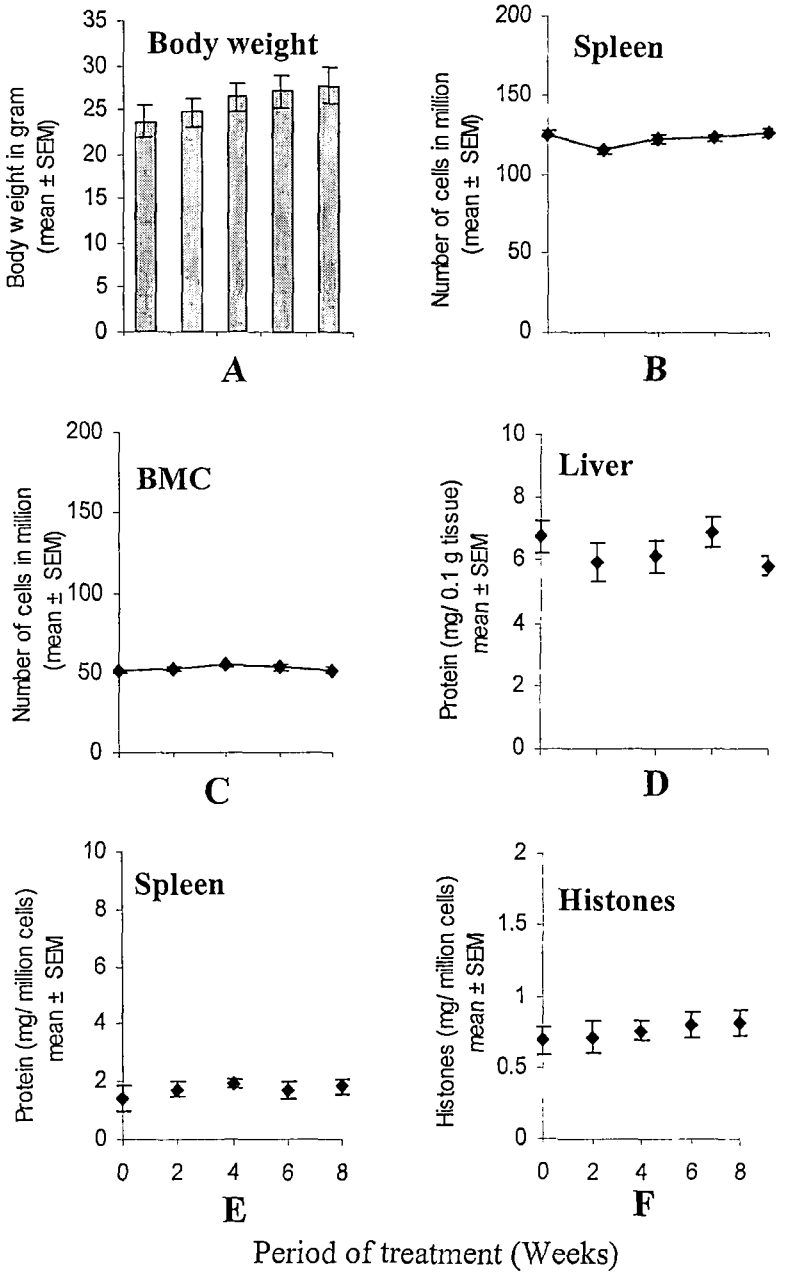

Figure 25: Effect of 3-aminobenzamide on general physiology of treated mice as compared to untreated (control) for a period of 8 weeks. Panel A: body weights; panel B: cell numbers in spleen; panel C: cell numbers in BMC; panel D: total protein contents in liver; panel E: total protein contents in spleen and panel F: histones content in spleen. Values represent mean of three independent experimental groups with two mice in each.

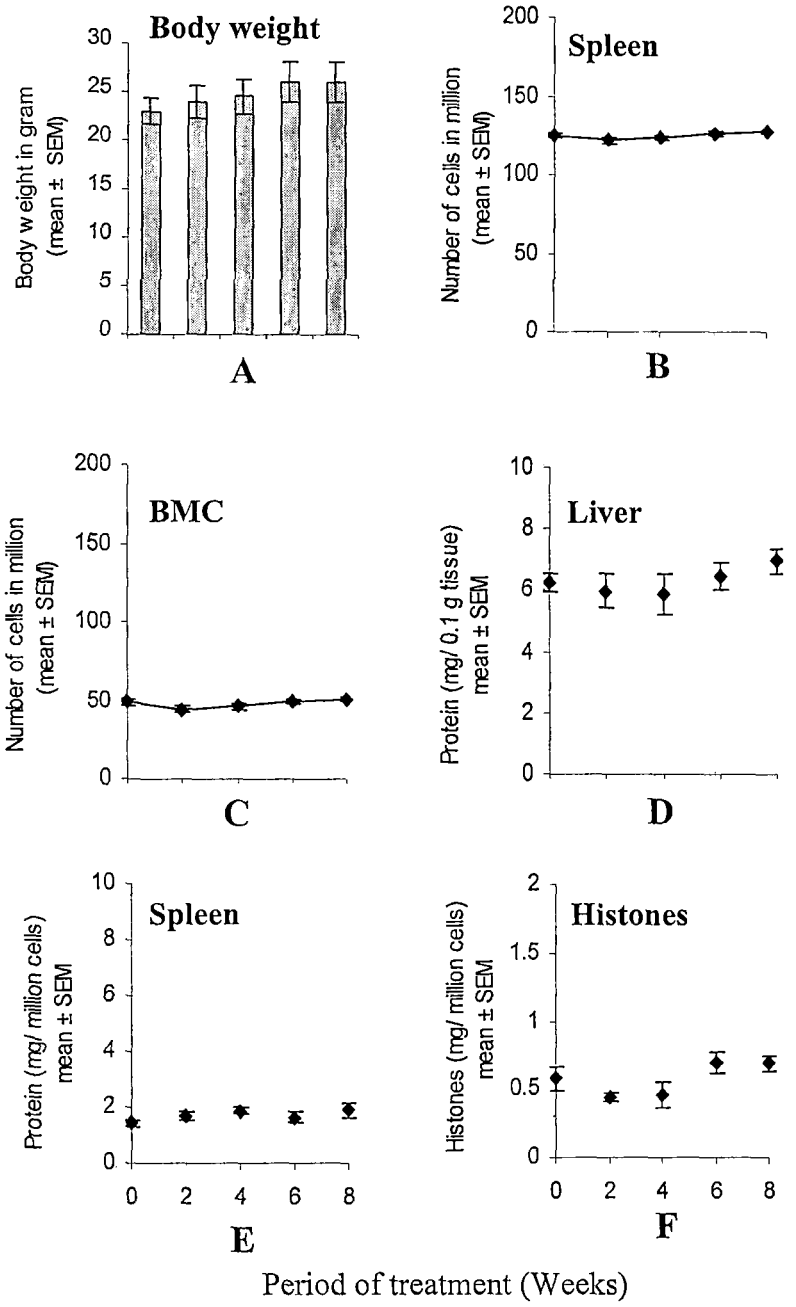

Figure 26: Effect of dimethylnitrosamine and 3-aminobenzamide in combination on general physiology of treated mice as compared to untreated (control) for a period of 8 weeks. Panel A: body weights; panel B: cell numbers in spleen; panel C: cell numbers in BMC; panel D: total protein contents in liver; panel E: total protein contents in spleen and panel F: histones content in spleen. Values represent mean of three independent experimental groups with two mice in each.

3.19 DNase I DIGESTION PATTERN OF CHROMATIN FOLLOWING DIMETHYLNITROSAMINE TREATMENT

DNase is a processing enzyme, which binds to DNA nonspecifically. DNase I digestion pattern was used as a parameter of chromatin structural modulation. Fig. 27 shows agarose

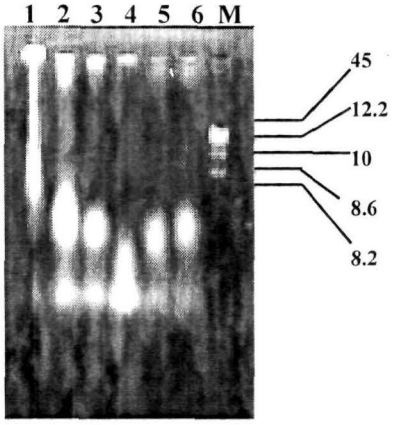

Figure 27: DNase I fragmentation pattern of chromatin isolated from spleen of untreated and DMN exposed mice. Lanes 1 and 2: normal chromatin without and with DNase I, lanes 3 – 6 with DNase I: 2, 4, 6 and 8 weeks, respectively. Enzyme action 2 min at 37 °C.

electropherogram representing the cleavage of enzyme DNase I in chromatin by 2 min digestion at 37°C after DMN exposure for a period up to 8 weeks.. The progressively higher degradation of chromatin by the enzyme reached maximum at 4 week time period. Thereafter there was resistance to chromatin degradation until the end of treatment period.

3.20 DNase I DIGESTION PATTERN OF CHROMATIN AFTER 3-AMINOBENZMIDE TREATMENT

Fig. 28 shows chromatin cleavage pattern induced by DNase I in 2 min at 37 °C following 3-AB treatment. The chromatin degradation by DNase I was much pronounced in this case after 6 weeks exposure to 3-AB.

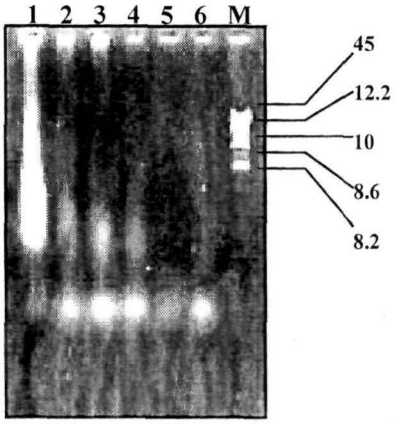

Figure 28: DNase I fragmentation pattern of chromatin isolated from spleen of untreated and treated mice with 3-aminobenzamide. Lanes 1 and 2: normal chromatin without and with DNase I, lanes 3 – 6 with DNase I: 2, 4, 6 and 8 weeks, respectively. Enzyme action 2 min at 37 °C.

3.21 CHROMATIN DIGESTION PATTERN BY DNase I FOLLOWING DIMETHYLNITROSAMINE AND 3-AMINOBENZAMIDE EXPOSURE

Fig. 29 depicts the chromatin degradation patter generated by DNase I under the combined effect of DMN and 3-AB.

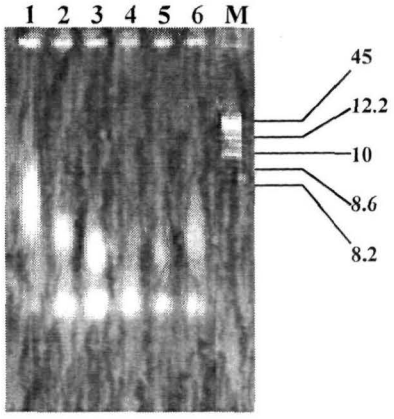

Figure 29: DNase I fragmentation pattern of chromatin isolated from spleen of untreated and treated mice with dimethylnitrosamine in conjunction with 3-aminobenzamide. Lane 1 and lane 2: normal chromatin without and with DNase I, lanes 3 – 6 with DNase I: 2, 4, 6 and 8 weeks, respectively. Enzyme action 2 min at 37°C.

The degradation of chromatin appeared to be more severe in this treatment condition. The cleavage of chromatin progressively increased till 6^{th} week. A slight resistance to degradation by DNase I was observed in 8^{th} week of exposure.

3.22 POLY-ADP-RIBOSYLATION OF TOTAL CELLULAR PROTEINS DURING ASCITES DALTON'S LYMPHOMA INDUCED TUMORIGENESIS

Level of total PAR was monitored in different tissues after ascites Dalton's lymphoma induction in mice for a period up to 15 days. Response of PAR level from different tissues following ascites induction was variant. Immunnoprobed blots with polyclonal antibody against ADP-ribose polymer are depicted by lane 2 of panel A, B, C and D and E of Fig. 30 while the amount of proteins loaded are shown by ink stained blots in each case (lane 1). Lane 2 of panel E depicts immunoprobed slot blot in ascitic cells histones in which PAR appears to be strongly inhibited. The analysis of ascitic cells histones started from 3rd day after ascites tranplantation.

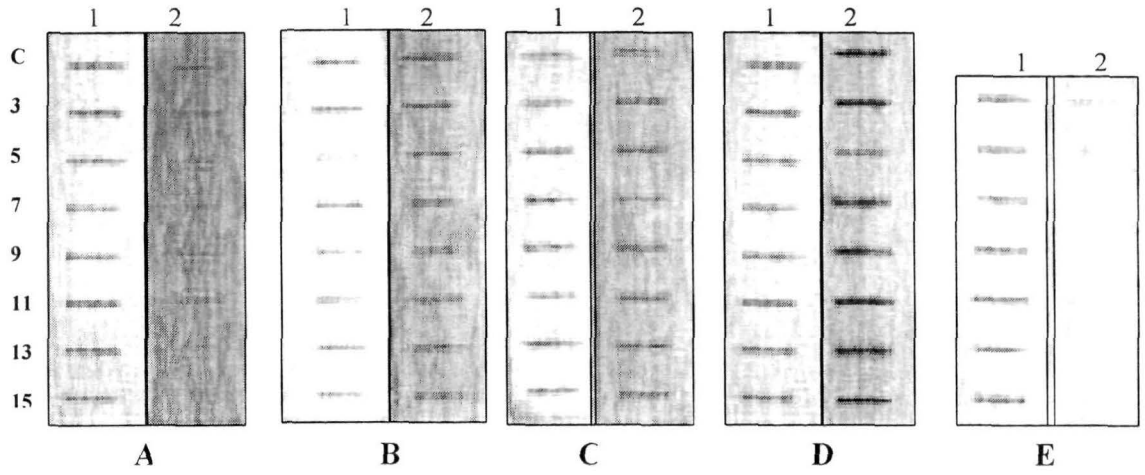

Figure 30: Total protein content and immunodetected proteins in various tissues of untreated and treated animals with ascites Dalton's Lymphoma induction as revealed by slot blot. Lane 1: Ink stained blot-indicating amount of proteins loaded and Lane 2: Immunodetection assay using polyclonal anti ADP-ribose polymer antibody, Panel A- isolated histones from spleen, panel B- liver homogenate; panel C- spleen homogenate and panel D- BMC homogenate and panel E- histones isolated from ascitic cells, blots from top to bottom indicates normal control, C and 3, 5, 7, 9, 11, 13 and 15 days respectively.

3.23 QUANTITATIVE ANALYSIS FROM SLOT BLOTS

Quantitative levels of PAR of total cellular proteins of different liver, spleen, BMC, histones isolated from both spleen cells and ascitic cells were studied during ascites Dalton's lymphoma induced tumorigenesis. Fig. 31 shows that level of PAR of total cellular proteins in most tissues remained invariant before and after the induction of ascites. However, time dependent non-significant slight changes of both lowering and elevation in the level of PAR during the treatment period were observed.

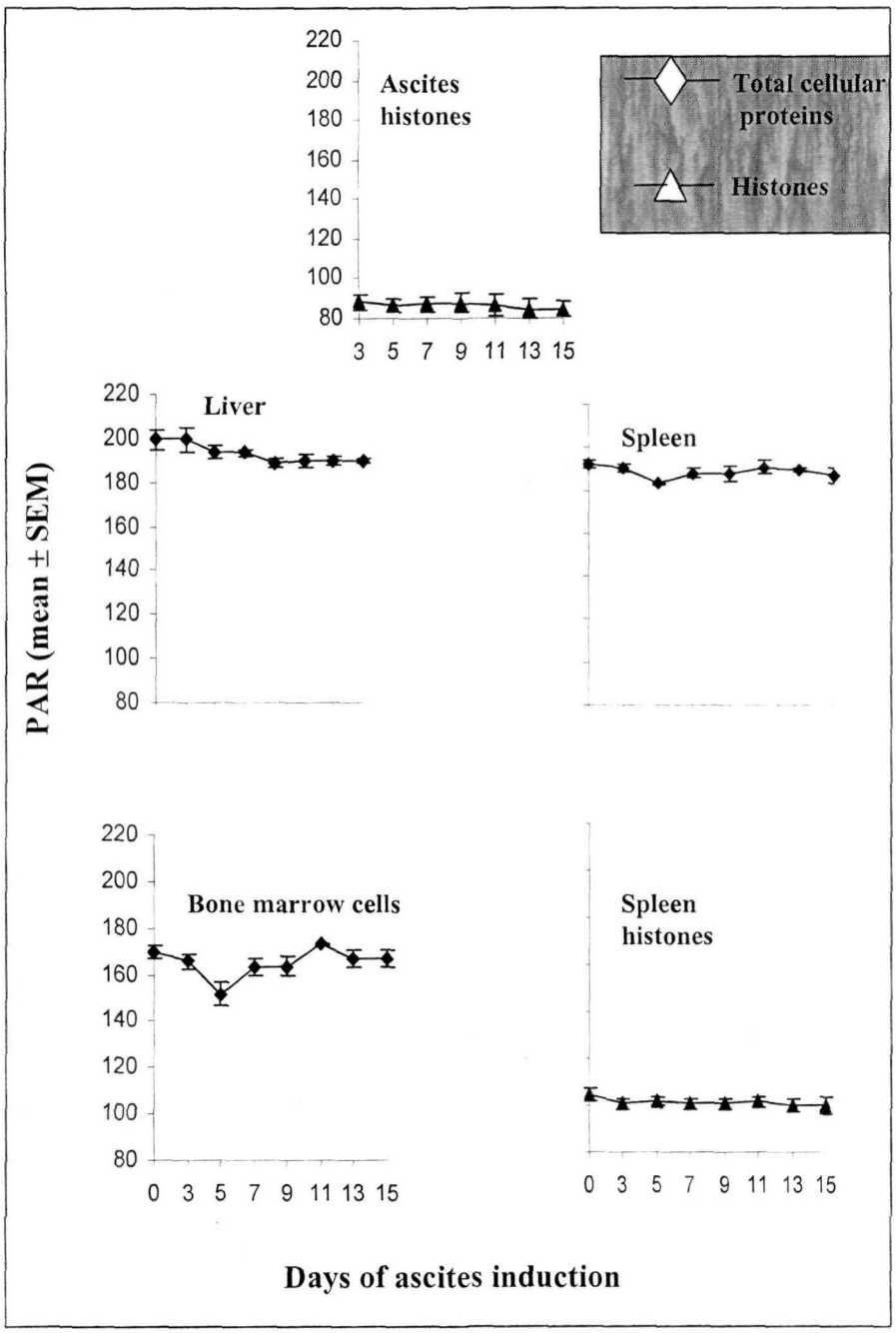

Figure 31: Levels of PAR of total cellular proteins of different tissues from unexposed (control) and those treated with ascites Dalton's lymphoma for a period of up to 15 days. Data (mean intensity) obtained from slot blot analysis. Values represent mean of three independent sets each with two replicates of slot blots.

3.24 HISTONE PROTEINS IN ASCITES DALTON'S LYMPHOMA CELLS

Fig. 32 shows electrophoretic profile (panel A) of histone proteins isolated from ascitic transformed cells. Panel B and C depict ink stained immobilized histone proteins and immunodetected proteins on nitrocellulose membranes after elctrophoretic transfer from SDS- PAGE resolved proteins. Among the histone proteins isolated on different days after ascites induction, it was apparent that many other proteins of high molecular weight also got isolated (panel A). The protein pattern showed over- or under- expression of some proteins. However, PAR reaction was very much inhibited as shown by panel C. Only core histones were weakly poly-ADP-ribosylated, which also appeared progressively inhibited as the growth of the ascites cells continued.

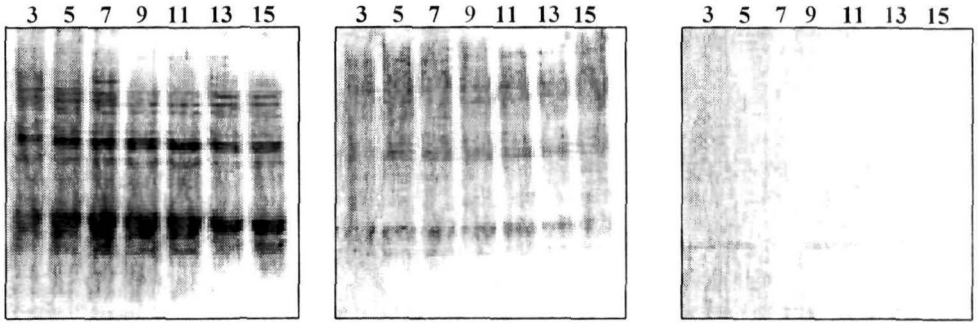

Figure 32: Histone proteins isolated from ascitic cells, Panel A - SDS- PAGE profile, Panel B- ink stain after electrotransblotting on to nitrocellulose memebrane, and Panel - C. immunodetection using polyclonal anti-ADP-ribose polymer antibody. Lanes left to right indicate 3, 5, 7, 9, 11, 13 & 15 days after ascitic cells induction respectively.

3.25 SDS-PAGE PROFILES OF PROTEINS AFTER ASCITES DALTON'S LYMPHOMA INDUCTION

The electrophoretic profiles of separated proteins by SDS-PAGE from histones isolated from spleen cells (panel A), liver (panel B) and spleen homogenate (panel C) after ascitc cells induction are shown in Fig. 33. During tumorigenesis induced by ascites Dalton's lymphoma, the protein expression pattern before and after the treatment of ascites induction was similar in all tissues examined except for slight over- or under- expression of some proteins as brought to light by the protein profiles shown below.

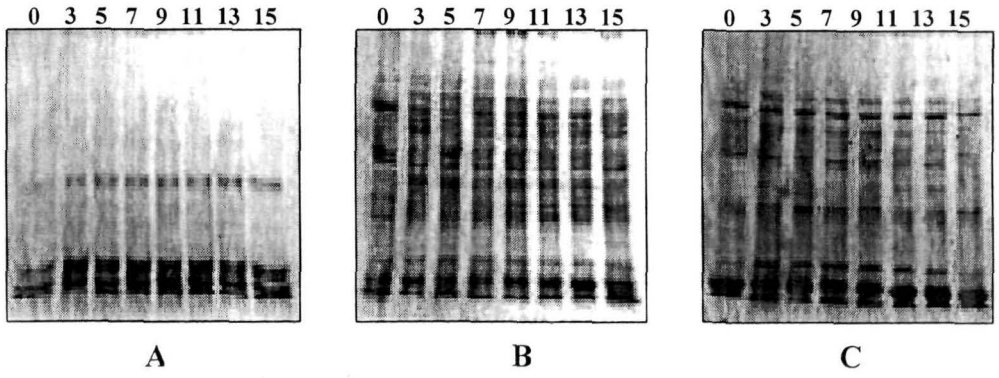

Figure 33: SDS-polyarcrylamide gel electropherogram of different tissues from normal and treated animals with ascites Dalton's Lymphoma for a period up to 15 days. Panel A - isolated histones from spleen cells; panel B - liver homogenate; panel C - spleen homogenate. Lanes left to right indicate control (0), 3, 5, 7, 9, 11, 13 & 15 days of treatment respectively.

3.26 INK STAINED WESTERN BLOTS OF PROTEINS FROM SDS-PAGE FOLLOWING ASCITES DALTON'S LYMPHOMA INDUCTION

Fig. 34 shows ink stained immobolized proteins of histones isolated from spleen (panel A), liver homogenate (panel B) and spleen cell homogenate (panel C). The separated individual proteins on the gel by SDS- PAGE were transferred on to the nitrocellulose membranes.

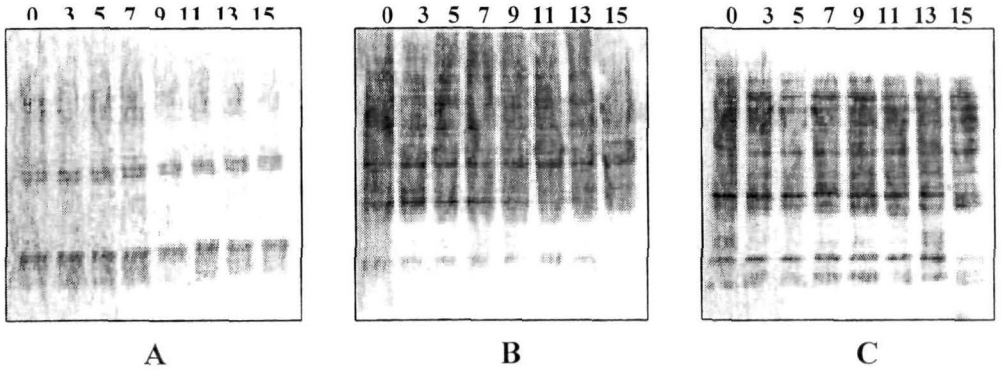

Figure 34: Ink stained Western blots on nitrocellilose membranes of SDS-PAGE gel electropherogram of different tissues from normal and treated animals with ascites Dalton's Lymphoma for a period up to 15 days. Panel A - isolated histones from spleen cells; panel B - liver homogenate; panel C - spleen homogenate. Lanes left to right indicate control (0), 3, 5, 7, 9, 11, 13 & 15 days of treatment respectively.

3.27 IMMUNODETECTED PROTEINS ON THE WESTERN BLOTS FOLOWING ASCITES DALTON'S LYMPHOMA INDUCTION

The SDS-PAGE separated proteins were immunoprobed on nitrocellulose membranes using polyclonal antibody against ADP-ribose polymer. The immunodetected blots of

isolated histones from spleen cells, liver homogenate and spleen homogenate are shown by panel A, B and C of Fig. 35 respectively. The results showed that histones were the main targets for poly-ADP-ribosylation. In homogenates of both liver and spleen, some proteins of high molecular weight were also ribosylated. The lowering of PAR was obvious in this case also.

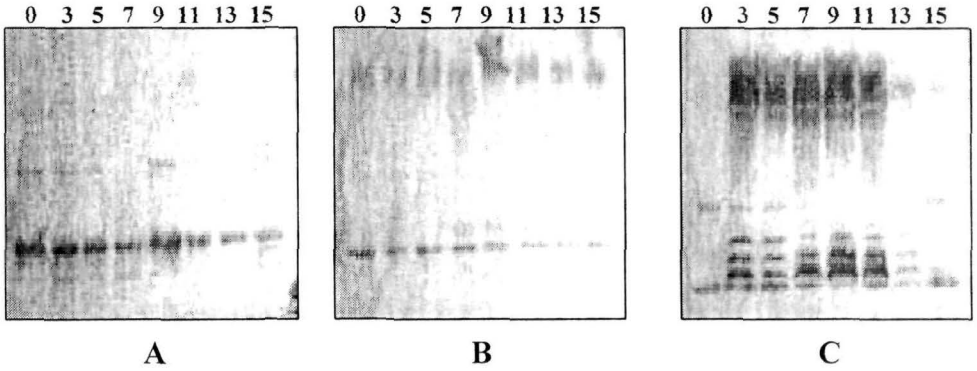

Figure 35: Western blot immunodetected nitrocellulose membrane of gel electropherogram of different tissues from normal and treated animals with ascites Dalton's Lymphoma for a period up to 15 days. Panel A - isolated histones from spleen cells; panel B - liver homogenate; panel C - spleen homogenate. Lanes left to right indicate control (0), 3, 5, 7, 9, 11, 13 & 15 days of treatment respectively.

3.28 QUANTITATIVE ANALYSIS FROM WESTERN BLOTS

Ascites Dalton's Lymphoma induced tumorigenesis in mice abdominal cavity was the subsequent target for studying PAR status. The experimental approach was similar with that of DMN induced carcinogenesis. Fig. 36 shows that exposure of mice to ascites Dalton's Lymphoma resulted in significant decrease in PAR in most of the histones in all the tissues analyzed with the progression of ascites tumorigenesis. PAR level was also reduced in histones isolated from ascitic cells.

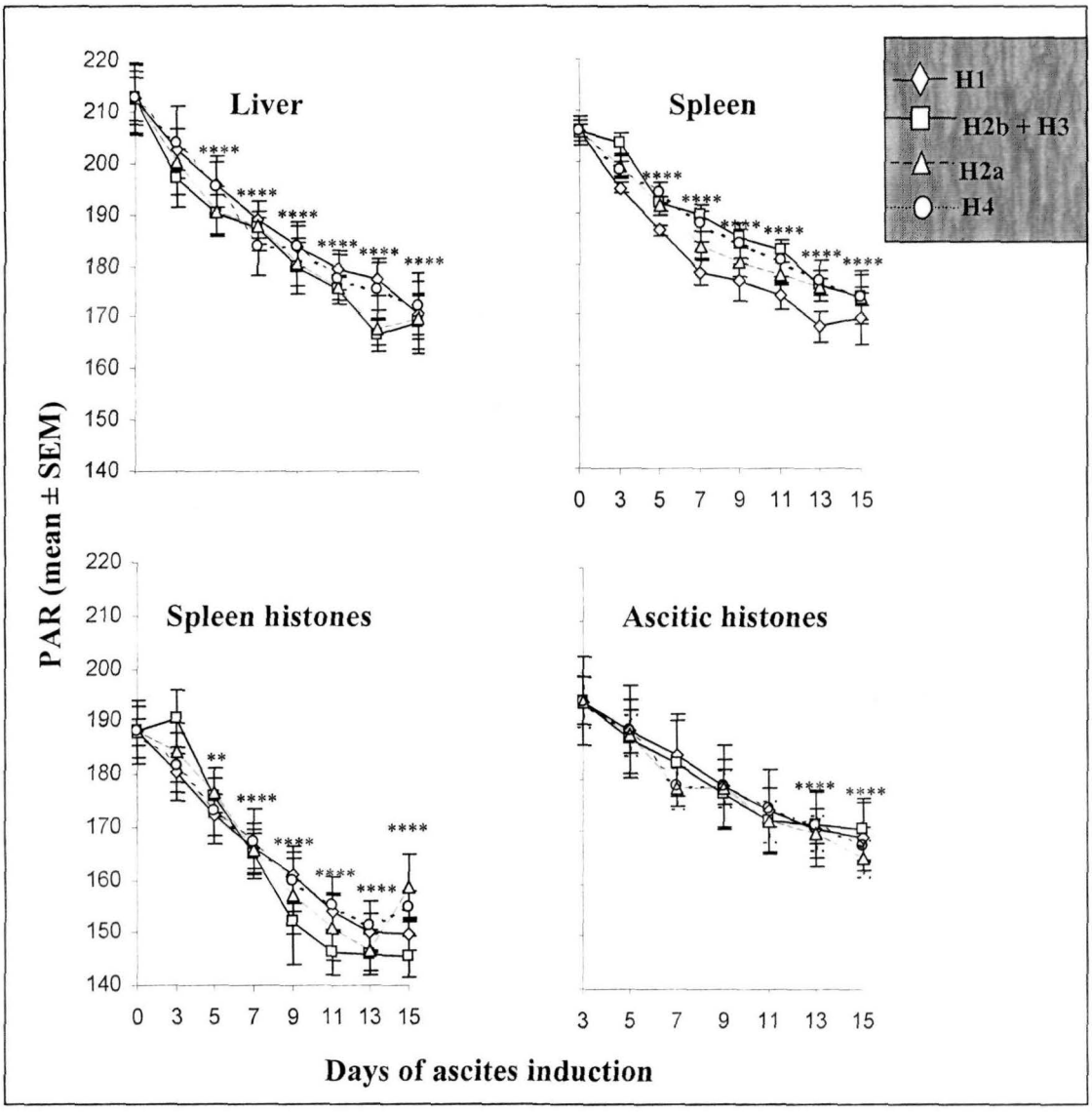

Figure 36: Levels of PAR of different histones from different tissues of mice treated with ascitic Dalton's lymphoma cells for a period up to 15 days. ****: significant decrease of level of PAR in all the histones H1, H3 & H2b, H2a and H4; **: significant inhibition of PAR in H1 and H3 & H2b. Data (mean intensity) obtained from three independent experimental sets each with two replicates of Western blots.

3.29 EFFECTS OF DALTON'S LYMPHOMA GROWTH ON GENERAL PHYSIOLOGY

3.29.1 Physical appearance

The tumor bearing mice survived till 21-23 days after transplantation. The tumor bearing mice were visibly different from the normal mice. Fig. 37 shows the enlargement of abdominal part (arrow head line) of the host mouse (B), as compared to a normal control mouse (A), due to accumulation of tumor cells.

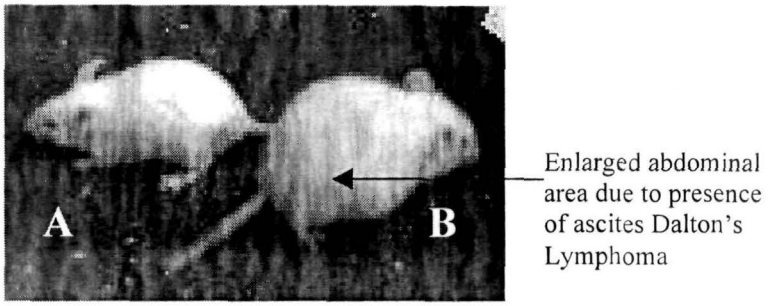

Figure 37: Photograph showing normal and 11th day old Dalton's Lymphoma bearing ascitic mouse. Abdominal cavity is enlarged (arrow) due to growth of tumor cells (B); a normal mouse (A).

3.29.2 Body weight of ascitic mice

Fig. 38 shows that the body weight of the tumor-bearing mice increased progressively during tumorigenesis.

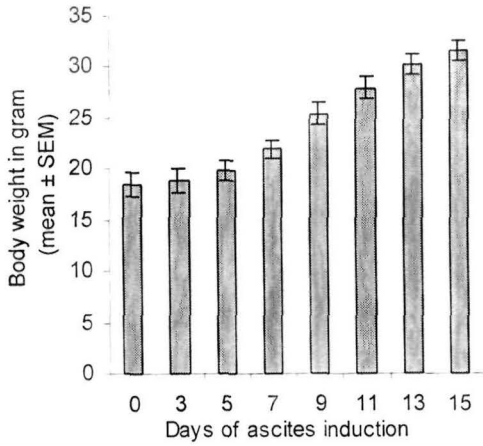

Figure 38: Body weight of normal and mice transformed by i. p. injection of 10^7 ascites Dalton's Lymphoma cells in the abdominal cavity. Experimental analysis was done until 15 day after transplantation of the tumor cells. Points represent three independent experimental sets each with two replicates.

3.29.3 Ascites cells growth

Similarly, the number of ascitic cells in the abdominal cavity of mice also increased progressively (Fig. 39).

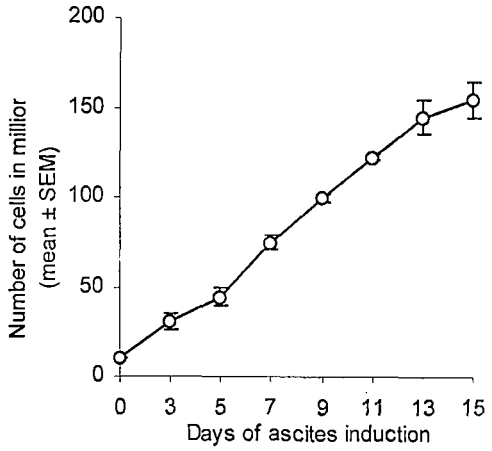

Figure 39: Ascites cells growth indicated by number of cells in the abdominal cavity of mice after i. p. transplantation of Dalton's Lymphoma for a period up to fifteen days. Points represent three independent experimental sets each with two replicas

3.29.4 Protein content

Other parameters like total protein contents in different tissues were also looked into. Fig. 40 depicts total protein content of liver and spleen cells of normal and ascitic mice after induction of Dalton's Lymphoma in the abdominal cavity of the animals.

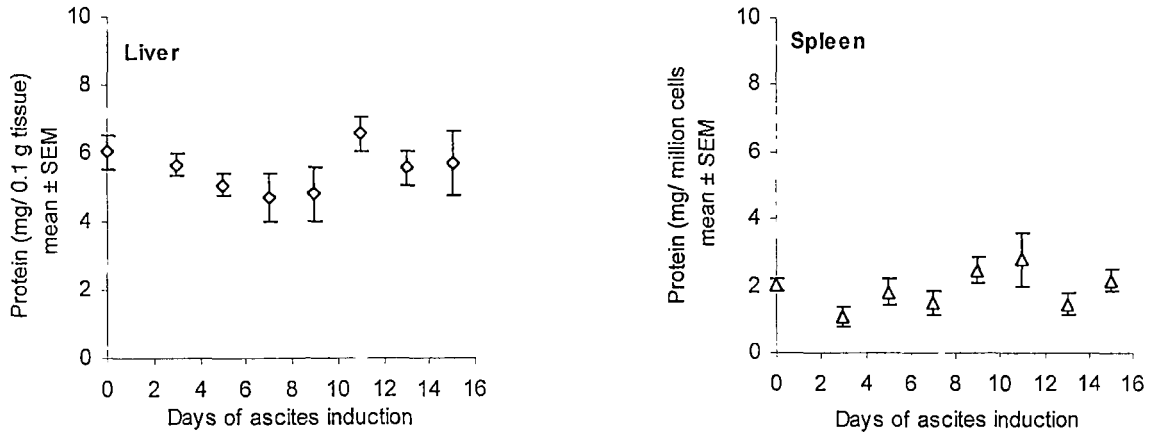

Figure 40: Total protein content of liver and spleen of normal and Dalton's Lymphoma induced ascitic mice for a period up to 15 days. Intraperitoneal transplantation of tumor cells into the abdominal cavity of animals was from 10^7 cells. Points represent three independent experimental sets each with two mice.

During the tumor growth period total protein content of both the tissues showed time dependent variations unlike that of animals under dimethylnitrosamine treatment.

3.29.5 Histone content

Similar pattern was observed in case of histones isolated from spleen (Fig. 41). Total histone content from ascitic transformed cells beginning from the day of transplantation

onwards was also looked into. There were changes in protein content during course of treatment.

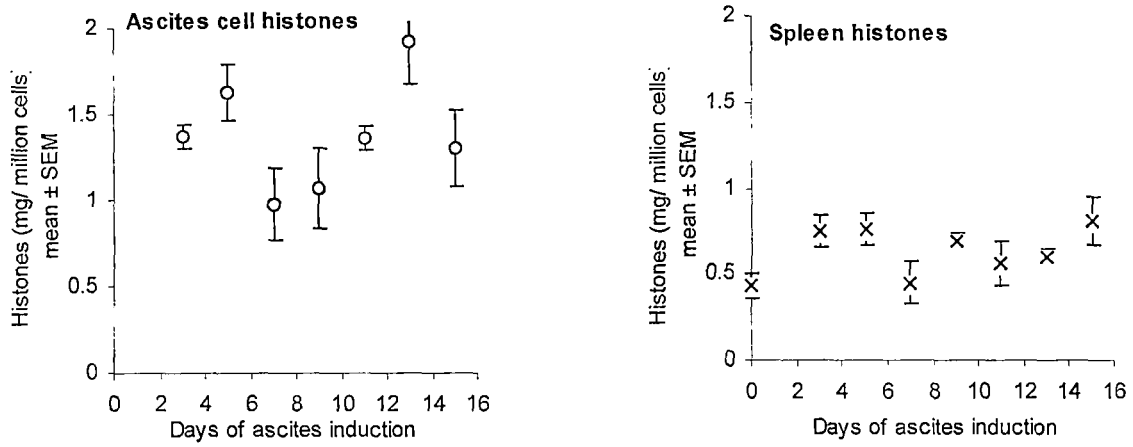

Figure 41: Total histone content in spleen and ascitic cells of normal and tumor (Dalton's Lymphoma) bearing mice. Points represent three independent experimental sets each with two mice

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CHAPTER IV

DISCUSSION

The connection between PAR of cellular proteins and various cellular processes, including carcinogenesis, have been under debate for many years. Various approaches have been utilized to understand the functions of this post-transnational modification of chromosomal proteins. Nonetheless, the PAR reaction and its implications remain enigmatic. Despite new developments in our understanding, numerous questions on the mechanism and role (s) of PAR are still to be answered.

Modification of nuclear proteins by poly-ADP-ribosylation involves sequential addition of ADP-ribose moiety of NAD^+ to acceptor proteins to form linear or branched polymers. It is also used as a means by various bacterial toxins to modify metabolic functions of the host. The existence *in vivo* of different types of ADP-ribose protein conjugates, their independent changes under varying metabolic or growth conditions and their uneven subcellular distribution indicate that the process of ADP-ribosylation serves multiple functions. In order to unravel the precise functions of PAR, it is indispensable to analyze the modification of specific acceptor proteins.

In this investigation, special emphasis is laid on the analysis of level of PAR of total cellular proteins and chromosomal proteins in chemically induced cellular transformation in mice. Among the acceptor proteins, histones have been studied most extensively among various PAR reactions (Althaus and Richter, 1987; Ueda and Hayaishi, 1985). It is known that 95% of PAR occurs in the nucleus (Lamarre *et: al.,* 1988; Saikia, 1996; Sharan *et al.,* 1998a; Yamanaka *et al.,* 1988), hence the importance of histones in this process. Besides, histones are crucial in the organization of chromatin superstructure. During pathological conditions like induced or spontaneous DNA damage, poly-ADP-ribose moieties are synthesized (Althaus and Richter, 1987). Two negative charges of two phosphate groups of ADP-ribose unit (Fig. 1) add to the charges of histones thereby, reducing the net positive charge. The electrostatic interactions between histones and DNA are, therefore, perturbed by the alterations of charge. The alteration in charge state of histones is proposed to contribute to relaxation of chromatin by the reduction in charge interaction between poly ADP-ribosylated histones and DNA. Degradation of the ADP-ribose polymers by PARG would have opposite effect and would induce chromatin recondensation (de Murcia *et al.,* 1988). The linkage of ADP-ribose polymers to histones are reported to be both covalent (Althaus and Richter, 1987; Boulikas, 1986; Neidergang, *et al.,* 1985) and non-covalent (Althaus, 1995; Nozaki *et al.,* 1994; Panzeter *et al.,* 1992).

Since histones serve as favorable primary targets of PAR, the process is considered as one of the unique post-translational modifications of histones.

The main enzymes involved in the ADP-ribosyaltion reaction are anabolic enzyme PARP and catabolic enzyme PARG. A balance exists between the anabolism and catabolism of poly- ADP-ribose in the cell, which determines the level of PAR (Sharan *et al.,* 1996). This specific equilibrium could change in response to a stress situation. Corresponding charge in chromatin superstructure with, thus, occur. Upon restoration of normalcy the equilibrium would be re-established. During initiation of carcinogenesis, the level of PAR has been shown to be altered (Patriat, 1997; Saikia, 1996) suggesting the implications of its role in DNA related cellular events of cellular transformation.

For endogenous quantification of ADP-ribose residues on proteins, it is crucial that methods designed to measure *in vivo* levels of ADP-ribose residues be reliable, sensitive and selective. The determination of endogenous level of poly-ADP-ribose still represents a laborious enterprise. One important feature of ADP-ribose polymer is very high turn over rate (Alvarez and Althaus, 1989). So, even slight changes in the level of PAR might have significant effect on the metabolism and other cellular processes associated with chromatin. The major difficulty in measuring poly-ADP-ribose is its extremely low level compared to related polymers such as nucleic acids in the cell (Ueda and Hayaishi, 1985). Earlier assay performed by ^{32}P NAD^+ incorporation into nuclear preparation or permeabilized cells are, therefore, of limited value compared to the quantification of endogenous conjugates (Saikia, 1996; Schneeweiss *et al.,* 1995; Sharan *et al.,* 1998b). Furthermore, the assay also had inherent interacting factors, which influenced the result of the assay (Schneeweiss *et al.,* 1995). An incorporation of isotopic method was developed wherein cell monolayer, grown *in vitro* in Leighton tubes, were used (Schneeweiss *et al.,* 1995). This new approach has helped identify and quantify common influences of intervening factors applicable to the conventional isotopic assay procedure and overcome them partially. Nonetheless, there are certain problems, which still continue to influence the modified isotopic assay and may give wrong values of PAR. This includes influences like, (a) cell permeabilization by hypotonic treatment, (b) cold shock or problems of uptake of labeled NAD^+ across the membrane as well as (c) change in cellular pools due to addition of labeled NAD^+. Above all, the isotopic assay cannot be used in all tissues, limiting the assay of PAR to cell suspensions, monolayer cultures or nuclei. Besides, it has

also been reported that ADP-ribosylation obtained *in vitro* differs markedly from the situation found in intact cells/tissues. Moreover, very scanty reports are available on *in vivo* work. For this reason, a reliable quantification of endogenous ADP-ribose polymer requires a highly sensitive test. Taking advantage of high antigenicity of ADP-ribose polymer (Sharan *et al.,* 1998b), an ELISA based assay using anti-poly-ADP-ribose antibody has been employed in this work for detection of endogenous poly-ADP-ribose level.

This ELISA based immunoblotting is a powerful technique, which has also been used to determine a number of important characteristics of protein antigens. For example, glycoprotein like gp 40 and gp 120 in patients infected with AIDS hev been characterised their presence, quantity, etc. In the assay developed and optimized in this work, level of PAR of total cellular protein and individual histones have been measured. Prior to probing blots for the presence of poly-ADP-ribose, the total composition of the proteins transferred on to nitrocellulose sheets was determined by staining with India ink (Section 2.16). This method of staining is cheap, reliable, sensitive and yields a permanent record. The staining method depends on the preferential adherence of the colloidal carbon particles of India ink to the immobilized proteins on the membrane. This process is employed for detecting proteins loaded either by slot blot or for those separated by SDS-PAGE and Western blotted on nitrocellulose membrane. Proteins were visualized within 1 hour. The assay of protein bound poly-ADP-ribose on Western or slot blots was determined by immunoprobing using anti poly-ADP-ribose polyclonal antibodies raised in the laboratory. The sequence of reactions in immunodetection, as outlined in the materials and methods (Section 2.15), was conveniently performed.

Earlier work done in our laboratory had shown that the sensitivity of polyclonal antibody to detect ADP-ribosylated proteins in cellular system is higher than that of monoclonal antibody (Sharan *et al.,* 1998b). Strongest favorable points of this novel technique are its simplicity, sensitivity and applicability to varied tissues without involving tedious sample preparations like those involved in radioactive assay. Apart from this, the method can be applied to any tissues unlike the earlier isotopic method which required to make cell suspension and was limited to only loosely bound cells such as cells of spleen and bone marrow. This assay neither needs a single cell suspension nor permeabilization. Any tissue can be homogenized and used for the assay of PAR.

Prior to monitoring level of PAR, the specificity of ADP-ribose polymer and polyclonal antibody, raised against it in the laboratory, was checked using Ouchterlony immunodiffusion assay (Section 2.9). There was distinct and strong cross reactivity between ADP-ribose polymer (antigen) and polyclonal antibody as demonstrated by a precipitin line formation (arrow in Fig. 5). On the other hand, no precipitin line developed between antibody and BSA (Fig. 5). To confirm the specificity of immune interaction between anti ADP-ribose polyclonal antibody and the ADP-ribose polymer antigen, snake venom phosphodiesterase (SVP) enzyme was employed (Section 2.10). Snake venom phosphodiesterase specifically splits the pyrophosphate bond of poly-ADP-ribose endonucleolytically (Sugimura, 1973) yielding PR-AMP (phosphoribosyl-adenosine mono phosphate) and 5' AMP released from the polymer terminus. In contrast, phosphodiesterase isolated from rat liver cleaves poly-ADP-ribose exonucleolytically, and also hydrolizes NAD^+, NADH, and ADP-ribose (Futai *et al.,* 1968). Hydrolysis proceeds from the AMP-terminus of each polymer to the bound protein and does not produce oligomers of PR-AMP (phosphoribosyl-adenosine mono phosphate). Phosphodiesterase isolated from tobacco cells also cleaves the pyrophosphate bonds in poly-ADP-ribose, ATP, NAD^+, inorganic pyrophosphate, cyclic nucleotides, dinucleotides (Shinshi *et al.,* 1976). For checking by the enzyme, ADP-ribose polymers associated with histones isolated from normal mouse spleen cells were degraded for 15 min at 37 ° C with SVP enzyme before slot blotting and immunodetection. Equal amounts of isolated histones were slot blotted on nitrocellulose membrane, which are shown by ink stained blots (Fig. 6 - panel A; lanes 1 & 2). Under the reaction condition specified (Section 2.10.1), SVP should specifically degrade of ADP-ribose moieties during the 15 min endonucleolytic cleavage. This could be confirmed by immunodetection following slot blotting. The nondegraded control sample showed intense PAR signal after colour developing (Fig. 6 - panel B; lane 1) while 15 min SVP treated sample showed very weak signal (Fig. 6 - panel B; lane 2) which was as negligible as the blank sample (Fig. 6 - panel B, lane 3). The results, therefore, confirm that the immunoassay of PAR, employed in this investigation, measured the level of PAR only.

For monitoring level of PAR during initiation of carcinogenesis, dimethylnitrosamine (DMN), a liver specific hepatocarcinogen, was used. A chronic exposure protocol was employed as it is known that administration of carcinogen at low doses over a protracted

period is more relevant to the biology of cancer in human than tumor induction by single dose protocols (Kleihues and Margison, 1976). The experimental examination was restricted to eight weeks at a chronic dose of 10 mg/ kg. b. wt. At this dose hepatocarcinogenesis has been shown to get initiated (Pariat and Sharan, 1998). DMN is found to be present in a large number of consumer items from beer to tobacco smoke to cosmetics (Hecht, 1997). It is also present in the acid environment of the stomach after ingestion of primary and secondary amines. It is reported to be present in high levels in fish and in salted fish. It is now believed that the endogenous production of nitrosamines explains particularly high incidence of gastric cancer in Japan and Iceland where salt preserved fish is a dietary common item (Mirvish, 1995). Therefore, it is apparent that the chosen carcinogen for investigation has easy access to humans through dietary and social habits. From these perspectives and practical reasons, DMN was chosen for this work for induction of carcinogenesis in mice.

Though carcinogenesis is a multistep process, this study was restricted to its initiation phase only as this phase is critical and irreversible (Pitot, 1989). DMN induced cellular transformation of liver in mice at the used dose and during this particular period of treatment. It has been reported earlier from this laboratory (Pariat and Sharan, 1995; Pariat, 1997). Since the process of initiation involves irreversible changes in the genome of the cell, it is important to understand events at this stage. However, the changes during early stage of carcinogenesis are usually at molecular level and do not manifest. After usually a long period, the physical manifestation becomes visible. But by that time metastasis may have started. Therefore, it is important that cellular transformation is detected at an early stage so that therapeutic intervention could be applied. In this light, the work was designed to look into change in the level of PAR of cellular proteins as well as chromosomal proteins during the onset of carcinogenesis and find a correlation. Effect of PARP inhibitor, 3- aminobenzamide (3-AB), was also monitored in order to determine the level of PAR in its presence in mice during DMN exposure. For the present study 2 mM of 3-AB was used which is reported to cause 96% inhibition of PARP activity (Sims *et al.*, 1982). Liver, spleen and BMC have been used for monitoring the levels of PAR. The level of PAR of histones isolated from spleen was also assayed.

GENERAL PHYSIOLOGICAL CHANGES AFTER DMN AND 3-AB EXPOSURE

During the investigation, in each experimental group, the physiological parameters such as body weight cell number in spleen and bone marrow, protein content in liver and spleen and histone content in spleen were monitored. Under DMN exposure condition, the numbers of spleen cells and BMC, protein contents in spleen cells, liver and isolated histones from spleen cells remained essentially invariant (Fig. 24). This suggest that under DMN influence and during the initiation phase (until 8 weeks), the overall physiological condition remained essentially unaffected except for the minor changes that could be related to normal growth and metabolism. Some mice (30%) showed signs of weakness and letharginess. This could be due to accumulation of DMN over the period of treatment. These observations are in line with the thinking that during initial phase of carcinogenesis, the above mentioned parameters remained invariant (Pariat and Sharan, 1995; Pariat, 1997; Pitot, 1986). Furthermore, the same parameters were also monitored when mice were exposed to 3-AB either alone or in combination with DMN. In both the cases, there were no marked differences between the control and the treated groups on the above parameters (Figs. 25, 26). This also indicates that the concentration of 3-AB (2 mM) used in the experiment did not have toxic effect on mice over a 8-week time period.

POLY-ADP-RIBOSYLATION (PAR) OF TOTAL CELLULAR PROTEINS AND HISTONES AFTER DMN AND 3-AB EXPOSURE

The level of PAR of total cellular proteins was monitored in liver, spleen and BMC of mice after exposing the mice to DMN (Section 2.4) using slot blotting (Section 2.13). Although the effect of DMN on level of PAR of total cellular proteins, as revealed by visible slot blots, was limited obscuring any clear insight, a clear picture emerged by quantification analysis. In order to quantify the levels of PAR, the mean intensities of the bands/slots were captured digitally by Kodak digital camera. The data obtained in each immunodetected slot for PAR were analyzed using analytical software, KDS 1D, and plotted. The graph depicts the effect of DMN on PAR of total cellular proteins in liver, spleen cells, BMC and isolated histones of spleen cells (Fig. 10). The results show that in liver, the target organ for induction of carcinogenesis by DMN, there was a non-significant tendency of increase in PAR level up to 4^{th} week followed by significant reduction in the later part of treatment. The statistically unchanged level of PAR suggests that synthesis

and degradation of poly-ADP-ribose were balanced during first 4 weeks of DMN exposure. However, in the later part of treatment, the catabolizing enzyme, PARG, could have overcome the anabolic activity of PARP causing lowering in level of PAR. In other tissues examined in this work, the level of PAR generally went down indicating that PARP was not activated or PARG was activated by DMN. The lowering of levels of PAR of total cellular proteins was significant only in liver and spleen. The level of PAR of isolated histone proteins from spleen cells also went down significantly in the later part (Fig. 10). The results also show that PAR of total cellular proteins (Fig. 7 -panels B; C & D; lanes 2) was higher than that of total histone proteins (Fig. 7 - panel A; lanes 2). This was evident even when higher amounts of histones were slotted on nitrocellulose membrane (Fig. 7 - compare lane 1 of panel A with that in panels B-D). This shows that other proteins than histones were also being modified by ADP-ribosylation.

Therefore, study of level of PAR of total cellular proteins did not provide a clear insight into the status of histone modification by this reaction during the initiation phase of carcinogenesis. To study the effect of DMN on different histones, separation of total cellular proteins into individual proteins by SDS-PAGE was carried out (Section 2.14.1). For comparison, isolated histones from spleen cells were also subjected to SDS-PAGE (Fig. 12). Because of methodological limitation in isolation of histones from liver, for this study histones isolated from spleen cells were analyzed. Since DMN was administered in drinking water to mice *ad libitum* for up to 8 weeks, all tissues should accumulate DMN. Therefore, it is assumed that the results obtained in spleen cells would represent all other tissues. The effect in liver is likely to be more pronounced than in spleen as liver, being a portal tissue, shall accumulate more DMN. The identification of different histone proteins was done on the basis of molecular weight and by comparing the bands with commercially available calf thymus histone marker. This criterion was applied to both isolated histones as well as whole homogenate preparations. The electrophoretic profiles of isolated histones were almost identical to standard histone markers (Fig. 11) indicating that the histones isolated from spleen cells contained all histone types, i. e. H1, H3, H2b, H2a and H4, for this proposed work. Because of overlapping bands of H2b and H3, both these histones have been analyzed together.

Under DMN influence up to 8 weeks, the protein expression pattern did not change markedly in liver and spleen cells homogenates and isolated histones from spleen cells

(Fig. 12 - panels A, B, C). However, slight under or over expressions of some proteins were apparent thereby, suggesting that change in the proteome was occuring during cellular transformation. Protein expression maps (PEM) are reported to be changed in several cancers. It has been shown that PEM of the normal human breast cells compared with the PEM of breast cancer cells helped to indentify the importance of proteins altered during cancer development (Page, 1999). Emmer-Buck (2000) studied protein profiles of normal and cancer cells by laser capture micro dissection and analyzed by two-dimensional polyacrylamide gel. It was shown that protein expression profiles and clinical data had correlation suggesting that this approach could be used to gain insight into the molecular aspects of cancer (Voss *et al.*, 2000). Eventhough the contribution of changes in selected proteins in carcinogenesis is not clear as yet, it is firmly believed that the proteomic analysis may provide clues to carcinogenesis process. Though this aspect has not been studied in this piece of work, such study will be of great relevance in the future.

The SDS-PAGE separated proteins of liver, spleen cells and isolated histones from spleen cells (Fig. 12) were processed for immunodetection (Section 2.15) after the proteins were electrotransferred onto nitrocellulose membranes by Western blotting. Equal amount of total proteins was loaded in each lane of the gel and one replica of the Western blotted nitrocellulose membranes was processed for ink staining (Section 2.16). The ink stained membranes in all treatment groups showed that the resolved proteins were efficiently transferred to nitrocellulose membranes (Fig. 13). The separated proteins were immunodetected using anti poly-ADP-ribose polyclonal antibody (Section 2.15).

The immunoprobed Western blots (Fig. 14) showed that essentially histone and some high molecular weight proteins, especially in spleen cells, were ribosylated. Other protein bands present on the Western blot (Fig. 13) were not ribosylated and hence, did not show band after immunodetection (Fig. 14). Upon quantification by mean intensity measurements of different histone bands it was clear that the extent of PAR of individual histones in liver, spleen cells and histones were different (Fig. 21, 22, 23). The observed differences in ribosylated histones could be attributed to different high turn over rate of poly-ADP-ribose (Alvarez and Athaus, 1989). PAR of histone H1, H2b + H3 and H4 in liver were significantly inhibited under DMN influence from 4^{th} week or later (Fig. 21). The same was not true for spleen cells except for histone H1 in the 6^{th} or 8^{th} week DMN exposure (Fig. 22, 23). These results suggest that DMN exposure selectively effected the

PAR of histones in liver, which also happens to be the target organ for DMN induced carcinogenesis (Pariat and Sharan, 1995). Slight increase in the level of PAR of total cellular proteins in liver (Fig. 10), therefore, must have been caused by high PAR of non-histone cellular proteins during that period. In the later period of DMN exposure, however, there was a decline of PAR for both histone and non-histone proteins in liver. This was different in case of spleen cells. Both whole homogenate of spleen cells and histones isolated from spleen cells exhibited lowering of PAR of histone proteins, albeit non-significantly, under the influence of DMN (Fig. 22, 23). The PAR of total cellular proteins in spleen also showed decline following DMN exposure (Fig. 10). Therefore, it can be derived from these results that in spleen the level of PAR manifested by total cellular proteins is essentially due to PAR of histones. The cause of lowering of PAR may be activation of PARG or inhibition of PARP activities (Fig. 1) because this reaction is a dynamic process. Thus, it can be derived that DMN influence may be manifested by the interplay of activities of the two enzymes of ribosylation reaction.

To address to this question, a potent inhibitor of enzyme PARP, 3-AB, was used in this investigation at a concentration such that nearly all PARP activity was inhibited (Sims *et al.,* 1982)). Mice were exposed to 3-AB by way of weekly injection at a concentration of 2 mM and effects of this treatment were monitored on PAR of total and individual proteins. The slot blot assay representing PAR of total cellular proteins (Fig. 8) shows, in general and as expected, relatively low intensity signal after immuno-probing (lanes 2) for all tissues in relation to amounts of protein slotted (lanes 1). Upon quantification this was confirmed, as lowering of PAR was evident, especially in spleen and bone marrow (Fig. 10). Liver exhibited significant inhibition only in the 8^{th} week after 3-AB treatment (Fig. 10). The level of PAR of different histones under 3-AB influence was monitored by Western blot analysis (Fig. 17). The quantification of PAR of different histones from liver and spleen under the influence of 3-AB revealed the exact extent of the effect. In liver the inhibition of PAR was extensive and significant, especially for H1, H2a and H2b + H3 (Fig. 21). On the other hand, in spleen homogenate (Fig. 23) or in isolated histones (Fig. 22), the inhibition of PAR of different histones under the influence of 3-AB were not so pronounced.

It is to be noted that 3-AB at the concentration of 2 mM, used in this investigation, is reported to cause nearly 100% inhibition of PARP (Sims *et al.,* 1982). Therefore, no new

PAR reaction of cellular proteins should occur in presence of 3-AB. However, PAR was still detectable in samples subjected to slot or Western-blot immunodetection assay (Figs. 8, 17). This may apparently appear contradictory to expectation. On considering the immunodetection assay, used in this investigation, it, however, becomes clear that the observations are not contradictory. The nearly complete inhibition of PARP, the main synthetic enzyme of PAR modification of a target protein, was caused only after 3-AB injections were given. This would halt new synthesis of ADP-ribose moieties on the target proteins. But the residual ADP-ribose moieties, attached to target proteins earlier than 3-AB exposure, would still be there on target proteins. The immunoprobing detects these polymers on the target protein in our assay giving relatively weaker but detectable bands (Figs. 8, 17).

The level of PAR of total cellular proteins and individual histones was monitored when mice were exposed to DMN in combination with 3-AB (Section 2.4 & 2.5). The combined treatment caused a progressive inhibition of PAR of total cellular proteins as revealed by slot-blot immunoassay (Fig. 9 - panels A, B, C, D; lanes 2). By visual examination also the inhibition was found to be more pronounced than that caused by DMN (Fig. 7 - panels A, B, C, D; lanes 2) but a little less intense in case of exposure to 3-AB (Fig. 8 - panels A, B, C, D; lanes 2). The Western-blot immunodetection, representing PAR of individual, mainly histone proteins, also show similar visual impression (Fig. 20). Upon quantification and plotting of the data the picture was clearer.

In terms of effect of combined treatment of DMN and 3-AB on PAR of total cellular proteins, spleen showed significant inhibition of PAR from 2nd week onwards while liver and bone marrow showed significant inhibition only from the 6th week after start of the exposure (Fig. 10). In liver and bone marrow cells, the combined exposure to DMN and 3-AB caused more pronounced lowering of PAR of total cellular proteins than that by DMN alone but less than that by 3-AB (Fig. 10). In spleen cell homogenate and isolated histones this was slightly different. The combined treatment caused more inhibition of PAR up to 6 weeks than either DMN or 3-AB alone (Fig. 10). In general these results suggest that the inhibitory effect of DMN on PAR of total cellular proteins was potentiated by combination of DMN with 3-AB. The degree of inhibition was, however, different for different tissues. The quantification data of PAR of individual histones from homogenate of liver, isolated histones and homogenate of spleen cells are plotted in Figs. 21, 22 and 23, respectively.

Liver, the target organ for DMN carcinogenesis, exhibited potentiation of inhibitory effect of DMN on PAR under the combined treatment regime (Fig. 21). PAR of histone H1 was very significantly effected. Histones H2a, H2b + H3 as well as H4 had similar trends, especially after 6 weeks of exposure. The lowering of PAR by the combined treatment was usually less pronounced than that caused by 3-AB exposure (Fig. 21). Spleen, on the other hand, produced slightly different results. Potentiation of DMN induced lowering of PAR was generally visible in mice exposed to the combined regime. But the extent of lowering of PAR caused by the combined treatment regime was more intense than 3-AB alone for histones H1, H2a and H4. Therefore, the results show that even in spleen, which is not a target organ for DMN induced carcinogenesis, potentiation of effect of DMN was caused by simultaneous presence of 3-AB.

The Western blot results show that most non-histone proteins of liver were not detectable by the immunoassay employed in this work (Figs. 14, 17, 20 - panel B) except in spleen where some non-histone protein bands were visible (Figs. 14, 17, 20 - panel C). Since the polyclonal Ab detects all poly ADP-ribosylated proteins in spleen including several high molecular weight non-histone proteins, it can be assumed that in liver non-histone proteins were not targets of PAR reaction. The possibility that some poly ADP-ribosylated proteins were not detected by the immunoassay in liver sample can be ruled out as such bands were visible in spleen samples. Moreover, since the extent of lowering of PAR under different treatment regimes for the PAR of total cellular proteins (slot-blots; Fig. 10) and individual histones (Western-blots; Figs. 14, 17, 20) were similar. Therefore, the result also supports the earlier suggestion that histone proteins, especially H1 and H2b, are the main targets for poly-ADP-ribosylation in liver and in spleen (Althaus and Richter, 1987; Saikia, 1996). Our investigation shows that different histones are modified by PAR variably in different tissues. Looking at the Western blots (Figs. 14, 17, 20), it is evident that degree of PAR of core histones was always higher than that of histone H1. This is in line with the result that histone H1, *in vivo* was found to be only a minor acceptor of ADP-ribose groups in rat liver and in hepatoma cells (Hilz *et al.,* 1983). In spleen cells, PAR of some high molecular weight proteins, other than histones, was also observed (Fig. 14, 17, 20 - panel C). Among high molecular weight proteins, PARP is reported to be automodified (Adamietz, 1987). This automodification causes inhibition of PARP enzyme activity, thereby, reducing the PAR of other target proteins or heteromodification (Buki *et al.,*

1991; Ferro and Olivera, 1984; Tanaka *et al.,* 1995). This explains why lowering of PAR of other proteins was caused especially in spleen as inactivated PARP will not be able to carry on the PAR anabolic reaction.

The potentiation of effect of DMN by 3-AB, reported in this piece of work, is similar to earlier report wherein 3-AB was found to potentiate the cytotoxicity of carcinogens depending on its concentration (Borek *et al.,* 1984). Concentration above 2 mM is reported to stimulate additional DNA damage leading to the death of the animals (Morgan, 1983). Although 3-AB resulted in inhibition of PAR, as expected, it seemed not to have any role in other cellular events. That the inhibitory effect of 3-AB did not cause any serious alterations to the cellular physiology is demonstrated by the SDS-PAG electropherogram of isolated histone proteins which show no perceptible change on PAGE analysis (Figs. 15, 18 - panel A).

The low level of PAR of histones is likely to relax chromatin (Sharan *et al.,* 1996). Since DMN does eventually cause liver cancer in mice (Pariat, 1996), it appears that DMN induced qualitative and quantitative changes in PAR of histones were just optimal for initiation of carcinogenesis. Our study shows that 3-AB potentiated DMN-induced inhibition of PAR (Fig. 21). This suggests that inhibitor of PARP, 3-AB, may affect the course of DMN-induced carcinogenesis by significantly lowering PAR of histone proteins in liver but not in other tissues.

STATUS OF CHROMATIN

The concept of chromatin as a dynamic environment for gene expression has emerged with more identification and characterization of protein complexes in nucleosomes (Jacobson and Pillus, 1999). Chromatin modification complexes such as PAR heighten the significance of this long-standing concept (Jacobson and Pillus, 1999). It has been speculated that the natural biopolymer ADP-ribose may be a ubiquitous cross-linking agent in eukaryotic chromatin. In order to look at the status of chromatin during the period of exposure to carcinogen or other agents, chromatin preparation from spleen cells of exposed mice were isolated (Section 2.17). The chromatin isolates were treated with DNase I to fragment the chromatin (Section 2.18.2). The results were analyzed by agarose gel electrophoresis (Section 2.18.3). The results show that under DMN influence, there

was increasing ease in fragmentation of chromatin by DNase I up to 4 weeks followed by slight resistance (Fig. 27). The exposure to 3-AB, on the other hand, provided greater ease of chromatin fragmentation by DNase I (Fig. 28). A combination of the two agents essentially showed a mid-path result wherein DNase I fragmented the chromatin more than DMN but less than 3-AB (Fig. 29).

DNase I fragments or degrades chromatin randomly depending on the availability of DNA cutting sites to the enzyme (Goodwin and Mathew, 1982). In a relaxed chromatin, the accessibility of DNA or its cutting sites to DNase I is more than that in its condensed configuration, thereby, this assay has been used as a convenient way of monitoring the superstructure of chromatin (Goodwin and Mathew, 1982; Schneewiss *et al.,* 2000). The ease with which chromatin is fragmented by DNase I, therefore, directly corresponds with state of relaxation of chromatin. The results of this investigation shows that chromatin was progressively moved into a relaxed configuration following exposure of mice to DMN up to 4 weeks after which it was relatively less relaxed (Fig. 27). In the first 4 weeks when chromatin became progressively relaxed, the DNA was progressively more available for interaction with the ultimate carcinogenic form of DMN (Boulika, 1992). After this period, of course, the relative condensation of chromatin was observed. So, it can be suggested that while in the first 4 weeks the chromatin DNA interacted with DMN or its metabolically activated forms (formation of adducts, etc.), in the 5^{th} and 6^{th} week it reduced the possibility of these adducts being removed as chromatin shifted back to relatively condensed state. This assumption seems attractive, as it is known that initiation of carcinogenesis is irreversible process (Pitot, 1986). Acquiring relatively more condensed configuration in the 5^{th} and 6^{th} weeks will prevent easy removal of adducts by different repair machinery (Althaus, 1992; Dantzer *et al.,* 1999; Satoh and Lindahl, 1992; Satoh *et al.,* 1994).

The combination of DMN and 3-AB exposure, in line with other results, showed potentiation of DMN effect as the chromatin was progressively more fragmented by DNase I up to 6 weeks (Fig. 29). There was, however, a slight resistance to DNase I fragmentation in the 8^{th} week. The exposure to 3-AB, on the contrary, showed higher ease with which DNase I fragmented the chromatin (Fig. 28) suggesting that 3-AB alone was not conducive to maintenance of chromatin structural integrity.

Enhancement of PAR causes relaxation of chromatin and vice versa (Section 1.7.1). This relationship was corroborated by assay of PAR of histone proteins based on widely used isotopic method (^{32}P-NAD^{+}). The present investigation on PAR, utilizing the immunoblot assay of PAR, however, gives opposite impression. The general lowering of PAR, especially of histone H1 and some core histones (Fig. 21, 22, 23), is accompanied by relaxation of chromatin superstructure as revealed by DNase I fragmentation (Fig. 27, 28, 29). This seemingly contradictory observation needs to be explained. The reason lies in the way PAR was assayed by isotopic and immunoblot assays. In the former, the assay revealed only the rate of incorporation of ^{32}P-NAD^{+} in permeabilized single cell suspension or nuclei. An increase in the rate of incorporation was interpreted as higher level of PAR and a decrease as lowering of PAR of target proteins (Aubin, *et al.,* 1983). The assay does not indicate the status of PAR prior to the assay. It can be illustrated by taking an example of two target proteins in which first protein has low level of PAR and the second protein has 2 times more of PAR prior to application of isotopic assay. Supposing that the first protein, for any reason, incorporated ^{32}P-ADP-ribose at a higher rate than the latter, then in autoradiogram, the first protein shall give a more intense band than the second one. This result shall be interpreted as high level of PAR for first protein and low for the second. Naturally this is not consistent with the real situation. In the immunoblot assay, on the other hand, the polyclonal antibody supposedly recognizes the epitopes on the poly-ADP-ribose attached onto the target proteins and not the rate at which the polymer is growing. Therefore, the assay is supposed to give a total quantitative measure of the polymer, which can be quantified. If the polymer is big, the intensity of band shall be high and reverse when the polymer was small. Thus, we firmly believe that the immunoblot assay actually gives the physiological measure of PAR of target proteins, which was not necessarily, so in the isotopic assay. Therefore, from the findings of this investigation, a model can be proposed to explain relaxation of the superstructure of chromatin by lowering of PAR of histones, especially H1, H2a and H2b + H3 (Figs. 21, 22, 23). The lowering of PAR of histones shall make the histones more electropositive as the histone protein shall have lower quantum of ADP-ribose attached to it. This shall cause stronger electrostatic interaction between histones (electropositive) and DNA (electronegative). This stronger interaction between H1 and linker DNA shall make the linker DNA piece more rigid. The same shall happen when PAR of core histones decrease. A relatively more rigid nucleosome will resist bending that is necessary for acquiring supercoiled state. Therefore, the chromatin shall be relatively more relaxed or, in other

words, relatively less condensed. In the reverse situation, wherein the PAR of histone proteins increases over their normal level, relaxation of chromatin shall result. But the cause of this relaxation is likely different. When PAR of histones increase, the net electropositive charge of histones shall decrease. This shall cause weaker interaction between the histones and DNA, thereby, facilitating relative relaxation of chromatin. Thus, it appears that for the optimal superstructure of chromatin to be maintained, optimum PAR of histones is necessary. Any deviation, either above or below the optimum level, shall lead to relaxation of chromatin.

The exposure to DMN caused lowering of PAR of histones in general. This lowering was further potentiated by the simultaneous presence of 3-AB. Under the influence, the chromatin acquired a relatively more relaxed structural configuration. Since the dose and protocol of administration of DMN, used in this work, are know to eventually cause carcinogenic transformation in mice, it is logical to assume that the relatively relaxed structure of chromatin was in favour of carcinogenesis. Since, the cause of relaxation of chromatin was the progressive lowering of PAR of histones, it may be derived that a negative correlation exists between level of PAR and cellular transformation by DMN.

GENERAL PHYSIOLOGY AFTER DALTON'S LYMPHOMA ASCITES INDUCED TUMORIGENESIS

Another system examined in this investigation was study of level of PAR in liver, spleen cells and BMC during intraperitoneally induced tumorigenesis by Dalton's lymphoma ascites in mice. It is expected that the continuous growth of ascites tumor in mice abdomen will perturb the normal physiology of other tissues. The mice usually survived for 21-23 days after transplantation of 10 million ascites cells. Therefore, this became a very convenient model to monitor PAR during tumorigenesis. Enlargement of the abdomen due to accumulation of tumor could be observed (Fig. 37). Monitoring the increase in ascitic cell number during its growth started from the 3rd day onwards as it was not practically feasible to take out ascites cells one or two days after transplantation. As normal cell could not be used as control for transformed cell, the ascites cell taken out from 3rd day was considered as control. A regular increase in tumor volume was noted with time following tumor transplantation. The metabolic condition of the tumor bearing mice appeared to be gradually affected and the animal died subsequently. This could be

related to the supply of nutrients necessary for continuous growth of tumor from the mice. In this investigation, the observations were made up to 15 days only.

Physiological parameters such as body weight, number of ascitic cells, protein content in liver and spleen cells, histone content in ascitic cells and spleen cells were studied during tumorigenesis induced by Dalton's lymphoma ascites. The number of ascitic cells during tumor growth increased as expected (Fig. 39). It was observed that the growth of ascitic cells was proportional to the increase in body weights of the animals bearing tumor (Fig. 38). In liver and spleen, during the tumor growth period (15 days observation), the protein content in liver and spleen showed declining tendency which was quite variable (Fig. 40) unlike those mice exposed to DMN treatment (Fig. 24). Histone content in ascitic transformed cells beginning from the day of transplantation was monitored (Fig. 41). There were changes in histone content during the course of treatment. The increase was more distinct in ascitic cell histones as compared to histones from spleen cells (Fig. 41). The variances observed could be due to the influence of tumor growth in the abdomen of the mice, which may show slight differences depending on the physiology of different mice. From the results obtained, it is apparent that during the period of tumrigenesis the changes in the physiological characteristics were distinctly different from normal control mice. It is known that during the later part of carcinogenesis changes in physiological characteristics become more obvious and this was observed in the setup using ascites model. In order to determine the level of PAR, as in DMN induced carcinogenesis, immunoblot assay was employed, PAR of both total cellular proteins of liver, spleen cells and BMC as well as isolated histones from spleen cells and ascitic cells were monitored.

POLY-ADP-RIBOSYLATION (PAR) IN TOTAL CELLULAR PROTEINS AND HISTONES

Equal amount of proteins from all samples were slot blotted, which is demonstrated by ink stained blots (Fig. 30 - panels A, B, C, D, E; lanes 1). It was observed that histones isolated from ascitic cells showed significantly weaker signal of PAR while homogenates of liver, spleen cells, BMC and histones isolated from spleen cells showed higher PAR signals. It has to be noted that level of PAR of histones isolated from ascites cells was monitored from 3rd day of intraperitoneal injection onwards. This was because only from

this, the number of ascites cell was sufficient to subject them to histone isolation (Section 2.12).

The results (Fig. 30) makes it very apparent that in the ascites cells PAR of histones was very much inhibited. Upon quantification of slot blots, the level of PAR of total cellular proteins of liver, spleen, BMC and histones isolated from ascitic cells and spleen cells remained essentially invariant through the period of investigation (Fig. 31). The physilogical level of PAR of cellular proteins of liver, spleen and bone marrow cells were relatively high. On the contrary, the extent of PAR of histones isolated from spleen and ascites cells were very low (Fig. 31). The SDS-PAGE profile of histones isolated from ascitic cells showed the presence of many other proteins of high molecular weight (Fig. 32 - panel A) unlike the histones isolated from from spleen cells (Fig. 33 - panel A). This suggests that in the ascites cells new proteins may have been expressed which were isolated along with histones. However, these proteins as well as were not ribosylated as revealed by immunoassay (Fig. 32 - panel C). The SDS-PAGE profile of proteins of liver and spleen showed that the pattern was similar with slight over or under expression following ascites induction (Fig. 33 - panels B, C). This was similar to that observed in case of DMN exposure.

Ink stained Western blots of liver and spleen homogenates on to nitrocellulose membranes showed the efficient transfer of proteins from SDS-PAGE gels (Fig. 32 - panels B; Fig. 34 - panels A, B, C). Immunoprobing the Western blots revealed that in liver and spleen homogenates, core histones and some high molecular weight proteins were targets of PAR (Fig. 35 - panels B & C) similarly as incase of DMN induced carcinogenesis. However, the isolated histones from ascites cells (Fig. 32 - panel C) as well as spleen cells (Fig. 35 - panel A) showed that only core histones were poly ADP-ribosylated. Visually one could see progressive lowering in the intensity of bands with progression of ascites tumorigenesis. Upon quantification, this was clear (Fig. 36). There was decline of PAR till the end of treatment period. Similar results were observed for PAR of histones isolated from spleen cells and ascitic cells. The lowering of PAR could either be due to inactivation of PARP and simultaneous activation of PARG or from activation of PARG while PARP enzyme activity remained unchanged. It is also possible that, PARP enzyme may be automodified, at least in spleen which shows high PAR of high molecular weight proteins (Fig. 35 - panel C). The finding corroborates the earlier reports which suggested

that in Ehrlich ascites tumor cells, N-terminal region of the polymerase might be blocked (Holtlund *et al.,* 1980). N-terminal tail domains of histones are the sites of post-transnational modification. Therefore, it is likely that there was no synthesis of ADP-ribose polymer in the transformed ascites cells. Histones isolated from spleen cells, however, got modified with ADP-ribose moieties indicating PAR signal after immunodetection (Fig. 30 - panel A). In general even during ascitic tumorigenesis lowering of PAR of total cellular proteins as well as that of histones especially core histones was evident.

In the light of the findings from this work, it is apparent that the implication of PAR in carcinogenesis is well founded. It has been reported that factors such as PAR, which potentially may modulate chromatin superstructure, thereby, affecting rate of induction as well as repair of mutations, could be a new and effective weapon against cancer (Service, 1994). Therefore, study of the level of PAR during carcinogenesis may be useful as a predictive assay for monitoring progression of carcinogenesis. The lowering of PAR of histone proteins can be an early marker of chemical induced carcinogenesis as well as tumorigenesis. PAR, thus, can be a molecular medicine against carcinogenesis.

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CHAPTER V

CONCLUSION

The findings obtained from this investigation can be concluded as follows:

- A polyclonal antibody against heterogenous ADP-ribose polymer antigen isolated from normal mouse spleen cells has been raised in the laboratory. An ELISA based immunoprobing method employing the polyclonal antibody has been established and optimized. The method specifically detects PAR of proteins by immune interaction between the raised polyclonal antibody and ADP-ribose polymer antigens.

- In the assay developed and optimized in this work, level of PAR of total cellular proteins and individual histones have been monitored by slot blot immunoprobing for total cellular protein while Western blot immunodetection for individual histone proteins. The novel assay developed has been found to be simple, sensitive and the assay can be applied to varied tissue without involving tedious sample preparations like those in isotopic assay. Thus, the assay provides an advantageous step ahead of the conventional method.

- In DMN induced carcinogenesis, the physiological parameters such as body weight, cell number in spleen and BMC, protein content in liver and spleen, and histone content in spleen remained essentially unaffected during the treatment period. Similar was the case in 3-AB treatment, so also in combined regime of DMN + 3-AB. However, in ascites Dalton's lymphoma induced tumorigenesis, the growth of tumor cells was proportional to the increase in body weights of mice. Protein contents in liver and spleen showed declining tendencies.

- The slot blot immunoassay reveals that the method is limited obscuring a clear insight. However, general lowering of PAR in liver and spleen under DMN influence was observed in the later part of treatment. Level of PAR generally declined for histones. During carcinogenesis and tumorigenesis, the extent of PAR of total cellular proteins of liver, spleen and bone marrow cells were relatively higher than histones isolated from spleen and ascites suggesting ribosylation of other proteins besides histones.

- Under the influence of DMN, the protein expression pattern did not change markedly in liver and spleen homogenates and isolated histones from spleen cells except for

slight over- and under- expression of some proteins. Influence of Dalton's lymphoma showed similar results during tumorigenesis.

- Histones were primary targets of poly-ADP-ribosylation as revealed by Western blot assay. Most non-histone proteins were not ADP-ribosylated in liver. Polyclonal anti ADP-ribose polymer antibody also detects higher molecular weight proteins particularly in spleen in both the systems suggesting that proteins other than histones were ribosylated in spleen.

- The general lowering of PAR, especially of histones H1 and some core histones is accompanied by relaxation of chromatin superstructure as revealed by DNase I fragmentation. The results suggest that a negative correlation exists between level of PAR and cellular transformation by DMN.

- Lowering of level of PAR was evident in histones H1, H2b + H3 and H4 under DMN influence especially in liver as shown by the Western blot analysis. 3-aminobenzamide (3-AB), an inhibitor of PARP enzyme, potentiated the effect of inhibition of PAR in the combined regime of DMN + 3-AB. In spleen, inhibition was observed in H1 only in the later part of DMN exposure. The inhibition of PAR was extensive and significant, especially for H1, H2a and H2b + H3 in liver under 3-AB influence. In spleen, the inhibition of PAR of histones was not so pronounced. However, the lowering of PAR by combined treatment was usually less pronounced than that caused by 3-AB exposure. During tumorigenesis, general lowering of PAR in most histones in liver, spleen, histones isolated from spleen and ascites cells were more evident. Therefore, the extent of PAR in different histones varied.

- The results obtained from the work undertaken in this investigation suggest that lowering of PAR is a hallmark during both initiation phase of carcinogenesis induced by DMN as well as tumorigenesis induced by Dalton's lymphoma ascites. Therefore, it can be proposed that employing Western blot immunoprobing assay for measuring endogenous poly-ADP-ribose, lowering of level of PAR can be used as predictive assay for detecting carcinogenesis and tumorigenesis.

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CHAPTER VI

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Curriculum Vitae

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Academic Qualifications:

Exam.	Division (%)	Board/ University
Intermediate	I (68. 9)	Board of Intermediate Education, Hyderabad
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Research publications/ reports

1. Sharan, R. N., Devi, B. J., Humtsoe, J. O. and Schneeweiss, F. H. A. (1998); Immunodetection of cellular poly-ADP-ribosylation. In: Sharan, R. N. (ed.) *Trends in Radiation and Cancer Biology*. International Co operation Bilateral Seminars series, vol. 29, pp. 240-243; Germany: Forschungszentrum Juelich GmbH.

2. B. J. Devi and R. N. Sharan (1999); The progression of carcinogenesis is influenecd by cellular poly-ADP-ribosylation of histone proteins *in vivo* as revealed by immunoblot assay. Abstract, In, *Radiation Research, 11th International Congress of Radiation Research (ICRR)*, vol. I, Moriarty, M., Mothersill, C. and Seymoour, C. (eds.), Dublin, Ireland.

3. B. J. Devi and R. N. Sharan (1999); Poly-ADP-ribosylation of chromosomal proteins in dimethylnitrosamine induced carcinogenesis in mice. Abstract, In, "Radiobiology 2000", Trivandrum, India.

4. B. J. Devi and R. N. Sharan (2000); Dimethylnitrosamine induced hepato-carcinogenesis in mice: Immuno-assay of poly-ADP-ribosylation, a potential tool for mass screening. Abstract, In IASL-APASL Joint Meeting, Fukuoka, Japan.

5. B. J. Devi and R. N. Sharan (2001); Effects of poly-ADP-ribosylation of chromosomal proteins and its inhibition by 3-aminobenzamide on dimethyl-nitrosamine induced carcinogenesis in mice. Abstract, In "20th IACR", Ahmadabad.

Conference/ Workshop participated

1. "International Conference on Radiation Biology: DNA Damage, Repair and Carcinogenesis & Indo German Satellite Symposium on Molecular Biology of Radiation Damage and Repair" held at Department of Biochemistry, North-Eastern Hill University, Shillong, India from 7-10 April, 1998.

2. "International Conference on Radiation Biology" held at Regional Cancer Centre, Trivandrum, India from 17-19 February, 2000.

3. "20th Annual Convention of Indian Association for Cancer Research (IACR)" at Gujarat Cancer & Research Institute, N. C. H. Campus, Asarwa, Ahmedabad - 380016, India from 19-21 January, 2001.

4. "Seminar on Applications of Radio-isotopes and Radiation Technology" held at St. Anthony's Auditorium, Shillong on 26-27 November 1999.