STUDY OF MOLECULAR CHARACTERISTICS OF CHROMOSOMES DURING CHEMICALLY INDUCED CELLULAR TRANSFORMATION

ABSTRACT

BY

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SUBMITTED IN
FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF
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Theirs

The fundamental objective of science is to make the human life qualitatively better, free of diseases and sufferings. Despite the fact that modern industrialized world has successfully eliminated infectious diseases, the major cause of death of last couple of centuries, fear of cancer as a killer disease has not subsided. Even in the new millennium with all encompassing developments in science and technology related to medical field including publication of human genome sequence, the epoch making feat of human endeavors, cancer continues to be one of the most deadly disease threatening mankind today. The scientific community worldwide is putting their mental, physical and financial resources to combat cancer. With economic development, increasing prosperity, better life expectancy and changing lifestyle this disease has firmed its grip on humans further. Cancer is a progressively fatal form of disease making it particularly different from other diseases. No specific drug has either been discovered for cancer nor seems likely in the near future. This is why cancer has always challenged the scientists worldwide and continues to do so. Cancer has been associated with the living world, particularly humans, since time immemorial. The problem of cancer lies in several special characteristics associated with it. For instance, (a) the causes of the diseases are vast and uncertain, (b) it is a progressive disease with long gestation time spreading over several decades in certain cancers, (c) no medicine has yet been discovered to successfully treat it and (d) even though it originates in a localized region, it spreads to otherwise healthy parts of the body even before one knows its existence. Cancer statistics of most of the advanced countries are available. In our country unfortunately, no accurate statistics on cancer incidence or death is available. The cancer registries in India are estimated to cover only about 4-5 % of the population (less than 1 % rural and about 15 % urban population). Nonetheless, the emerging trend in India is no different than anywhere in the world, irrespective of the socio-economic factors. This suggests that the intensity of problem is much more in India since its population is significantly bigger than most developed/developing countries.

Obviously, a great deal of research and investigation are needed to combat the menace effectively. It is estimated that improved screening of population suspected to be exposed to carcinogens and consequent early detection of carcinogenesis can lead to a significant reduction (up to 35 %) in cancer death with the present knowledge of cancer therapy. Research aimed directly at cancer prevention and promoting use of available knowledge for cancer prevention is highly desirable in the present state of cancer control. Attempts are being made to identify suitable biomarkers, locate and map gene so that it may serve as critical tools in different areas of biological and medical research covering carcinogenesis. Gene or chromosome mapping can provide valuable information, which, in turn, can provide tools for diagnosis, understanding and treatment of cancer.

Mapping of chromosomes, however, is an expensive and elaborate process and its application requires sophisticated and expensive laboratory set-ups. This limits the possible application of such information to mitigate the problem of a large segment of human population in developing and under-developed countries including India. An alternative to these limitations is to characterize the chromosomes and the genes located on them in terms of their biochemical activities controlling cellular metabolisms. There are several advantages of this approach. To start with, by looking at an appropriate biochemical parameter, the effective consequence of any change in

gene in terms of mutation, over- or under-expression can be directly measured. The information provides the actual biological consequence of change(s) in the gene. Secondly, biochemical characterization of a gene of interest gives information on its activity irrespective of type of change(s), thereby, providing the real *in situ* status of the gene(s) and its biological consequences. Furthermore, it becomes a quick way of looking at the status of gene(s) while genetic mapping is time consuming and requires sophisticated laboratory set-up. In addition, this kind of molecular marker of carcinogenesis has possible applied potentials for diagnosis of cancer as well as for monitoring progression of cancer therapy.

Considering the advantages associated with biochemical characterization of chromosomes, the work was designed to look into the activities of gene(s) associated with poly-ADP-ribosylation (PAR) reaction by monitoring PAR of cellular proteins in mouse tissues. Mouse chromosome 14 houses the main anabolic enzyme for PAR, poly ADP-ribose polymerase (PARP) gene located at 14q11.2-q12. The main catabolic enzyme of PAR, poly-ADP-ribose glycohydrolase (PARG), has also been tentatively mapped on the same chromosome but its precise position on chromosome 14 has not been worked out.

Using a hepatocarcinogen, dimethylnitrosamine (DMN), and a naturally occurring general carcinogen, aqueous extract of betel nut (AEBN), in a chronic *in vivo* administration protocol on Swiss albino mice, this work has attempted to characterize changes on the genome or chromosomes of the mice during initiation stage of carcinogenesis. DMN has been shown to initiate cancer in mice in about 4 weeks of exposure. The characterization of the chromosomes was done by monitoring structural changes in the mouse genome using DNase I. Further characterization was done at biochemical level by monitoring poly ADP-ribosylation (PAR) of cellular proteins by an ELISA-based immuno-probe assay developed in the laboratory. Total PAR of cellular proteins as well as PAR of individual proteins, including isolated histone proteins, were monitored in liver, spleen cells (SC), bone marrow cells (BMC) and blood lymphocytes. The extent of total PAR of cellular proteins as well as that of histones was quantified densitometrically.

The mouse SC genome underwent progressive relaxation starting from 2nd week of DMN or AEBN administration as indicated by results of DNase I digestion of the genome. Analysis done by agarose gel electrophoresis and pulse-field gel electrophoresis produced similar, confirmatory results. DMN caused relatively more relaxation of the genomic DNA than AEBN under similar conditions. Concurrent administration of 3-aminobenzamide (3-AB), an inhibitor of PAR reaction, with DMN further relaxed the genome suggesting involvement of PAR in the process.

For biochemical characterization, PAR of cellular proteins was monitored using a novel ELISA-based immuno-probe assay developed in the laboratory. The assay is simple, sensitive, reliable, cost-effective and environment friendly since it does not involve use of any radioisotope. Total PAR of cellular proteins was assayed by slot-blot immuno-probing while PAR of individual proteins was assayed by Western-blot immuno-probing. The hepatocarcinogen DMN affected the total PAR of cellular proteins in liver, the target organ, as well as other tissues such as SC, BMC and blood lymphocytes. AEBN also showed similar results in all cases. Statistically significant and progressive lowering of total PAR of cellular proteins in the four tissues studied

was caused by DMN or AEBN almost immediately after exposure of mice to the carcinogen in a chronic oral administration protocol. DMN in combination with 3-AB, on expected lines, further inhibited the total PAR of cellular proteins in al tissues examined.

Tissue specific differences were apparent and the extents of inhibition of total PAR of cellular proteins were different in different tissues and for the two carcinogens used in this investigation. Histones proteins were found to be the main target proteins for PAR in all tissues beside some other high and low molecular weight proteins. Histone proteins were grouped as H1, H3/H2b and H2a for the analysis. PAR of these histones were variable in different tissues. However, in all cases, DMN or AEBN lowered the extent of their PAR progressively during initiation stage of carcinogenesis. Concurrent presence of 3-AB with DMN further lowered the PAR of histone proteins on expected lines. This suggests that DMN or AEBN affected gene activities or enzyme activities of PAR reaction in a way that PAR was lowered.

Blood lymphocytes mirrored the effect for both DMN and AEBN in terms of total PAR of cellular proteins as well as PAR of individual proteins, particularly histones. Lowering of PAR was statistically significant in all cases beyond 2^{nd} week of administration. The effect of two different carcinogens, DMN and AEBN, on the PAR of blood lymphocyte proteins may provided us with a handy tool for monitoring biochemical or physiological status of individuals exposed to carcinogens. This may possibly be a good biomarker for early detection of cancer or for monitoring progress of carcinogenesis since (1) it is a common post-translational modification associated with many proteins and (2) the assay, employed in this investigation, detects only the ADP-ribose moieties and not a particular protein.

All tissues examined in this investigation have shown a negative, nearly straight line, correlation between period of DMN or AEBN exposures and PAR of proteins from different tissues of Swiss albino mice. The most significant observation made in this study that has direct implication on its use in medical practice is that even blood lymphocytes exhibited significant reduction in PAR modification of cellular proteins with progression of DMN or AEBN exposure periods. This was in line with the trend exhibited by liver, SC and BMC. Both total PAR of cellular proteins and PAR of individual histone proteins showed similar trends. Different tissues or proteins did show variability in terms of PAR, but the general message was clear. Tissue specific variations were found which is normally expected. Though obtaining liver, SC or BMC from mice is no problem but the same is not true for human situation. The results from mouse blood lymphocytes give convincing clues that the strategy can be easily extrapolated to human situation. Further, immuno-probe assay of total PAR of cellular proteins is relatively simple as compared to the assay of PAR of individual proteins. The former involves slotting the sample directly on a NC membrane (slot-blot) and immuno-probing while, the latter, requires electrophoresis, transblotting the proteins from gel onto a NC membrane (Western blotting) and then immuno-probing. Since our results show a very clear, reproducible and statistically significant correlation between total PAR of blood lymphocyte proteins and initiation of carcinogenesis induced by two different classes of chemical carcinogens, DMN and AEBN, (Figs. 11, 13 and 14), it is logical to postulate that this slot-blot immuno-probe assay can be employed in human situation also. It is postulated, based on the results

presented in the thesis using mouse model that this strategy shall apply to any type of human cancer as well.

The biochemicals required for the whole slot-blot immuno-probe assay can, in principle, be packaged into a kit making it very convenient for transport. Most of the constituents of this kit have good shelf-life and require ordinary refrigeration, *etc.* The 1st Ab is the critical constituent, which actually detects the PAR modifications of proteins. In the investigation, the 1st Ab was raised against heterogeneous ADP-ribose polymer obtained from mouse SC. Preliminary investigation on human blood lymphocytes using the same 1st Ab against mouse ADP-ribose polymer shows that the 1st Ab was also able to detect PAR of proteins in human blood lymphocytes. This already supports the basic premise of application of the assay for detection of human cancer and for its use as a tool of mass screening. However, all procedures shall need to be standardized for assay involving human samples before final conclusion is drawn. Much remains to be done before it is applied to detect human cancer. Nonetheless, the work carried out and elaborated in the thesis takes us closer to the goal of making cancer detection simple, sensitive and reliable in the near future.

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Thesis

The North-Eastern Hill University
October 2003

I, **Lakhan Kma**, 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 the 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.

This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Biochemistry.

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PROF. R. N. SHARAN
Supervisor

Dedicated to

My family

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SYMBOLS AND ABBREVIATIONS

ADP	Adenosine diphosphate
AEBN	Aqueous extract of betel nut
Ag	Antigen
Ab	Antibody
3-AB	3-Aminobenzamide
AMP	Adenosine mono-phosphate
BCIP-NBT	5-Bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium
BMC	Bone marrow cell
BSA	Bovine serum albumin
°C	Degree Celsius
CBB	Coomassie brilliant blue
CML	Chronic myeloid leukemia
Da	Dalton
DDT	Dichloro-diphenyl-trichloro ethane
DTT	Dithiothretol
DEN	Diethylnitrosamine
DMN	Dimethylnitrosamine
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DPA	Diphenylamine
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbant assay
g	Centrifugal force
g	Gram
h	Hour
ha	Hectare
HMG	High mobility group
Kb	Kilobase
Kbp	Kilobase pair
kDa	Kilo Dalton
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrolase
µg	Micro gram
µl	Micro litre
Mbp	Megabase pair
min	Minute
ml	Millilitre
mM	Millimolar
mA	Milliampere
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
NC	Nitrocellulose
nm	Nanometer
PAR	Poly-ADP ribosylation
PARG	Poly-ADP ribose glycohydrolase
PARP	Poly-ADP ribose polymerase
PVDF	Poly-vinyl difluoride
RNA	Ribonucleic acid

RT	Room temperature
SC	Spleen cell
SCE	Sister chromatid exchange
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second
SPC	Sucrose-potassium phosphate-calcium chloride (buffer)
TBS	Tris buffered saline
TEMED	N, N, N′, N′-tetramethylethylenediamine
TTBS	Tween-20 tris buffered saline
UV	Ultra-violet
V	Volt

CONTENTS

Chapter 1

INTRODUCTION

The fundamental objective of science is to make the human life qualitatively better, free of diseases and sufferings. Despite the fact that modern industrialized world has successfully eliminated infectious diseases, the major cause of death of last couple of centuries, fear of cancer as a killer disease has not subsided. Even in the new millennium with all encompassing developments in science and technology related to medical field including publication of human genome sequence, the epoch making feat of human endeavors, cancer continues to be a most deadly disease threatening mankind today. The scientific community worldwide is putting their mental, physical and financial resources to combat cancer (Levi, 1999). With economic development, increasing prosperity, better life expectancy and changing lifestyle this disease has firmed its grip on humans further. Cancer is a progressively fatal form of disease making it particularly different from other diseases. No specific drug has either been discovered for cancer nor seems likely in the near future. This is why cancer has always challenged the scientists worldwide and continues to do so.

1.1. CANCER

Cancer has been associated with the living world, particularly humans, since time immemorial. The oldest recorded evidence of cancer is bone cancer found on the fossilized skeleton of dinosaur in Wyoming dating back to Triassic, Cretaceous and Jurassic periods, much before humans came into existence (Moodie, 1923). The first recorded evidence of human cancer has been found in the remains of Stone Age man (Tertiary period) in form of bone cancer of jaw bone (Brothwell and Sandison, 1968). Elaborate descriptions of cancer-resembling diseases of humans are recorded in old Indian scripts (*Vagabhata* and *Bhavamista*) as well as Mesopotamia before Christ, Roman and Greek literatures, and Bible (Neuberger and Pagel, 1902; Wise, 1860). Numerous writings on cancer and its treatment belong to the Middle Ages, from Renaissance and Baroque periods (Avicenna, 1593).

Cancer is normally associated with old age with possible exception of leukemia and bone cancer (Srivastava, 1999). The problem of cancer lies in several special characteristics associated with it. For instance, (a) the causes of the diseases are vast and uncertain, (b) it is a progressive disease with long gestation time spreading over several decades in certain cancers, (c) no medicine has yet been discovered to successfully treat it and (d) even though it originates in a localized region, it spreads to otherwise healthy parts of the body even before one knows its existence (Cairns, 1978). The monoclonal origin of cancer is widely accepted now. Cancer may be initiated from within by activation of oncogenes located in the genome or inactivation of tumor suppressor genes (Hanahan, 1986). It is also triggered by a host of exogenous factors, which include radiations, chemicals and other physical agents (Ladik and Forner, 1994). Every second chemical compounds seems to have its implication in cancers and the list is ever increasing (Castegnaro and Sansone, 1986). Cancer is likely to afflict up to one in three persons before their 75^{th} birthday in developed countries and one in four persons will die from it (Coleman, 1995). In the UK, almost a quarter of a million new cancers are diagnosed each year, and there are some 1,60,000 deaths (Coleman, 1995). Unfortunately no such accurate statistics on cancer incidence or death in India is available. The cancer registries in India are estimated to cover only about 4-5 % of the population, which includes less than 1 % rural, and about 15 % urban population (Srivastava, 1999). Nonetheless, the emerging trend in India is very similar to that of developed countries. This suggests that the

intensity of problem is much more in India since its population is significantly bigger than most developed countries. Obviously, a great deal of research and investigation are needed to combat the menace effectively.

The frequency and lethality of cancer underlie the persistent public concern about this group of diseases. Hence, there is great interest in any news of progress in cancer therapy as well as early detection of cancer. The facts also give some idea of the magnitude of the public health challenge which cancer now represents in developing and developed countries (Coleman, 1995). Many of the causes of cancers have been identified (Tomatis *et al.*, 1990) and many continue to be added to the list. It is generally agreed that research into discovering avoidable causes of cancer is now of utmost importance (Breslow and Cumberland, 1988). It is estimated that improved screening of population suspected to be exposed to carcinogens and consequent early detection of carcinogenesis can lead to a significant reduction (up to 35 %) in cancer death with the present knowledge of cancer therapy (Levi, 1999; NCI, 2002). Research aimed directly at cancer prevention and promoting use of available knowledge for cancer prevention is highly desirable in the present state of cancer control.

1.1.1. Carcinogenesis

A major feature of all higher eukaryotes is its defined life span or mortality with differentiation. This characteristic property extends to the individual somatic cell, whose growth and division are highly regulated (Kerr and Harmon, 1991). A notable exception is provided by cancer cells, which arise as variants that have lost their normal growth controls (Millar, 1995). Their ability to grow and propagate indefinitely or acquire immortality with dedifferentiation in inappropriate locations is hallmark of cancer (Millar, 1995). In relation to cancer development or carcinogenesis, the question of cells escaping from the normal homeostatic behavior in multicellular organisms is of prime importance. The mechanism of regulation of normal multicellular behavior seems to bear the answer to this. The control of homeostasis *in vivo* is dependent on the constant replenishment of old and damaged cells. This should occur in the absence of immunological stimuli through different lineage in the system. Defect in either stem cells or committed progenitors of each lineage that can proliferate as well as differentiate can lead to imbalances in the cellular homeostasis. This can lead to different malignancies.

1.1.2. Genetic basis of carcinogenesis

The concept of cancer as genetic diseases is widely accepted. The recognition of familial retinoblastoma and familial polyposis coli as heritable cancer syndromes have strengthen this concept. Similarly the observation of chromosomal abnormalities in neoplastic cells has contribution to the concept. A gross change in the genetic material, particularly non-random chromosomal abnormalities such as translocations is the chief features of many human tumors (Millar, 1995). Approximately, 90 % of patients with CML have the Philadelphia (PH^1) chromosome (Nowell and Hungerford, 1960) which results from the reciprocal translocation of DNA between chromosome 9 and 22. Aberrant proliferation of cells and abnormal growth is an important feature of most of the cancers. The various mechanisms, which might become disordered in malignant cell and result in abnormal proliferation, could be (a)

abnormalities in growth factor production, (b) abnormalities in growth factor receptors, (c) disturbances of post-receptor signaling and (d) reduced production of or sensitivity to growth inhibiting factors (Millar, 1995). In general, these abnormalities could be recognized at behavior level *in vitro* by loss of anchorage dependent proliferation, lack of density dependent growth inhibition and the secretion of excess or abnormal proteins (Millar, 1995). Demonstration of the ability of chemical to act as a carcinogen is directly related to its ability to bind and to mutate DNA has contributed to this concept further (Patterson and Yarnold, 1995). It has become apparent now that human tumors arise as a result of accumulation of mutations in essentially two classes of cellular genes. They are proto-oncogenes, sometimes referred to as dominantly transforming oncogenes, and tumor suppressor genes, also referred to as recessive oncogenes (Cooper and Lane, 1984; Horwich, 1995). Proto-oncogenes are normal cellular genes which when activated by mutation or over expressed act to promote tumor formation. The phenotypic effect of the activated allele is 'dominant' at the cellular level. This is because the mutation acts to up-regulate the activity of the gene. Tumor suppressor genes are normal cellular genes that promote differentiation and regulate proliferation. Loss of this function is pertinent to tumorigenesis. Inactivating mutations or deletions of both alleles of a tumor suppressor gene is required before gene function is completely lost. Because mutations in single alleles of tumor suppressor genes are relatively silent at the cellular level they have been termed recessive oncogenes (Patterson and Yarnold, 1995).

In general, at molecular level, carcinogenesis is recognized as a complex multi-step process. It has been suggested that the process involves several events at molecular level including mutations of different kinds, structural alterations, gene rearrangements, gene amplifications, altered and neo-gene expressions, *etc.* (Pitot, 1986). The process of carcinogenesis has three distinct stages (Pitot *et al.*, 1991). They are,

Initiation: This is a vital and irreversible stage in which multiple mutations in genetic material accumulate. The process involves relatively small structural changes such as the alkylation of specific bases resulting in single base transversion and small deletions in many genes (Milligan and Archer, 1988).

Promotion: It is the next step in the process of carcinogenesis and it is reversible. In this stage of carcinogenesis there is propagation of the initiated cells. The stage is characterized by reversible alteration in the gene expression.

Progression: This is an irreversible stage of carcinogenesis. There are visible alterations in the genetic material of the cells, which result in karyotypic instability.

1.2. RATIONALE AND SCOPE OF THE WORK

Justifiably, the emphasis on understanding the molecular and genetic bases of carcinogenesis has taken center stage across the globe. Attempts are being made to locate and map gene so that may serve as a critical tool in different areas of biological and medical research covering carcinogenesis. Gene or chromosome mapping can provide valuable information, which, in turn, can provide tools for diagnosis,

understanding and treatment of cancer. A lot of cancer research has been dependent on the small laboratory animal, albino mouse, which has acquired a center stage in our fight against cancer (Berns, 1999). The research has generated or continues to generate a large wealth of information in cancer diagnosis, prevention and treatment, thereby, contributing significantly to our fight against human cancer (Berns, 1999). The mouse (*Mus musculus*) genome consists of 20 pairs of chromosomes including a XX or XY sex chromosome pair (Fig. 1).

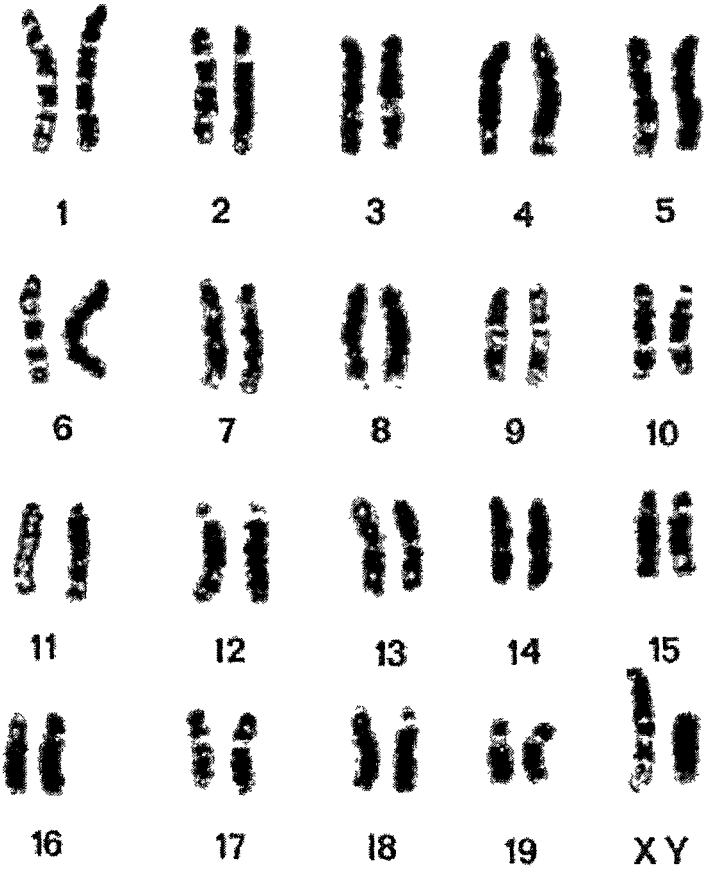


Figure 1: Normal *Mus musculus* karyotype. A complete diploid set of metaphase chromosomes from the laboratory mouse is shown. This karyotype was kindly provided by Dr. Muriel Davisson, Jackson Laboratory, Bar Harbor

Mapping of chromosomes are done in several ways. They are (a) linkage mapping, (b) chromosomal mapping and (c) physical mapping. In principle, all three approaches should provide the same information on the order of genes, distances between them and their status of activities. However, the relative distances that are measured by each mapping methodologies can be significantly different. While the physical map is taken as a more accurate description of the actual length of DNA and distances between genes, linkage and chromosomal mappings are not. This is not necessarily because the later two methods of mapping are inaccurate. Rather, they represent versions of the physical map that has been modulated according to different parameters (INTSYS, 2003). Mapping of genes may provide valuable clues to diagnosis and therapy of cancer in the future. However, it is an expensive and elaborate process and its application requires sophisticated and expensive laboratory set-ups. This limits the possible application of such information to mitigate the

problem of a large segment of human population in developing and under-developed countries including India.

An alternative to these limitations is to characterize the chromosomes and the genes located on them in terms of their biochemical activities controlling cellular metabolisms. There are several advantages of this approach. To start with, by looking at an appropriate biochemical parameter, the effective consequence of any change in gene in terms of mutation, over- or under-expression can be directly measured. The information provides the actual biological consequence of change(s) in the gene. Secondly, biochemical characterization of a gene of interest gives information on its activity irrespective of type of change(s), thereby, providing the real *in situ* status of the gene(s) and its biological function. Furthermore, it becomes a quick way of looking at the status of gene(s) while genetic mapping is time consuming and requires highly sophisticated laboratory set-up. In addition, this may serve as a molecular marker of carcinogenesis and may have desirable applied potentials for diagnosis of cancer as well as for monitoring progression of cancer therapy. It is to be noted that search for appropriate molecular and biochemical markers for early cancer diagnosis has great relevance to the present approach of our fight against cancer since by early diagnosis cancer mortality and morbidity are likely to reduce significantly (Miller *et al.*, 1990; Cuzick, 1999). It is also to be noted that study of cancer cases by looking into the proteomics profiles of patients has yielded valuable clinical correlation between them (Voss *et al.*, 2000; Emmert-Buck *et al.*, 2000).

Considering all the advantages associated with biochemical characterization of chromosomes, the work embodied in this thesis was designed to look into the activities of gene(s) associated with poly-ADP-ribosylation (PAR) in mouse genome. Mouse chromosome 14 (Fig. 2) houses the main anabolic enzyme for PAR, poly

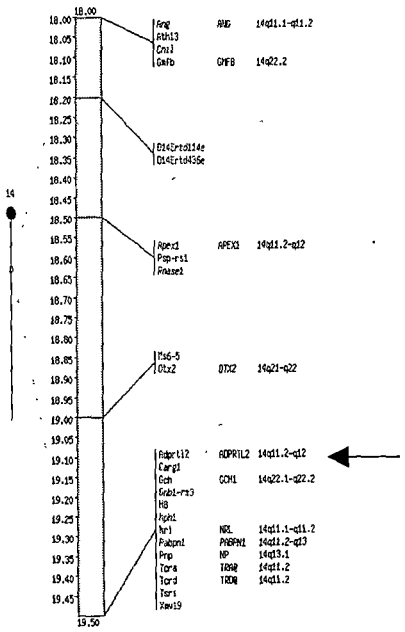


Figure 2: Genetic map of a part of mouse chromosome 14 showing location of some of genes that have been mapped on it, including the main poly-ADP-ribosylation reaction genes, poly ADP-ribose polymerase (PARP) (arrow) and poly ADP-ribose glycohydrolase (PARG).

ADP-ribose polymerase (PARP) gene (Adprt12; human gene equivalence - ADPRTL2) located at 14q11.2-q12 (MGI, 2003). The main catabolic enzyme of PAR, poly-ADP-ribose glycohydrolase (PARG), has also been mapped on the same chromosome but its precise position on chromosome 14 has not been worked out (MGI, 2003).

As has been discussed above, carcinogenic transformation is a complex multi-step process. It has been suggested that the process involves several events at molecular level including mutations of different kinds, structural alterations, gene rearrangements, gene amplifications, altered gene expressions, *etc.* during initiation, promotion and progression phases (Pitot, 1986). Many of these events such as the structural alterations, gene activation, *etc.* can be visualized indirectly by DNaseI digestion pattern of genomic DNA representing the total chromosomes. It is well known that if genes are active, the DNA is in relaxed conformation and *vice-versa* (Saikia, 1996; Pariat, 1997; Devi, 2001). During carcinogenesis the chromosome undergoes relaxation as has been observed in our laboratory (Saikia, 1996; Pariat, 1997; Devi, 2001) and elsewhere (Bellard, *et al.*, 1989). In the biochemical approach to characterize chromosomes the transcriptional and translational end products of the genes *i. e.* the proteins have been used in this work. The metabolic activity of these proteins or enzymes specific to poly-ADP-ribosylation (PAR) of cellular proteins was monitored. Attempt has been made to explore the possibility of its use as a diagnostic tool for early detection of cancer.

1.3. POLY-ADP RIBOSYLATION (PAR)

Proteins are the essential component of any living system. Once synthesized, proteins usually require specific modifications in order to become biologically functional. The modifications, commonly called the post-translational modifications, are very specific to proteins depending upon their final location and function in the cell. Post-translational modifications of proteins include acetylation, phosphorylation, methylation, adenylation, poly-ADP-ribosylation, *etc.* (Moldave, 1985).

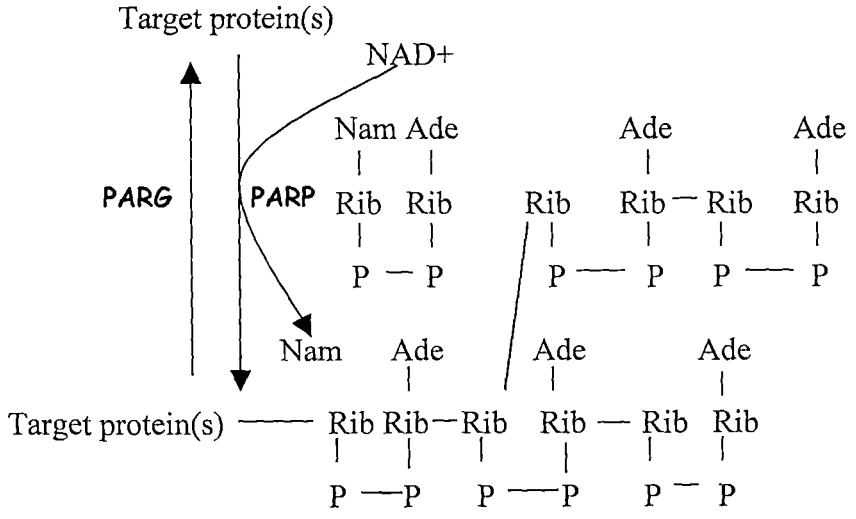


Figure 3: Schematic view of poly-ADP ribosylation (PAR) reaction in eukaryotes. Nam: nicotinamide; Rib: ribose sugar, Ade: adenine; P: phosphate; PARP: poly ADP-ribose polymerase; PARG: poly ADP-ribose glycohydrolase.

Poly-ADP-ribosylation (PAR) is a post-translational modification of mainly chromosomal proteins (Althaus and Richter, 1987). PAR is an enzymatic and reversible cellular reaction. It involves the transfer of ADP-ribose moieties from endogenous nicotinamide adenine dinucleotide (NAD^+), the substrate for the reaction, to a target proteins creating a linear or branched polymer of ADP-ribose moieties. ADP-ribose moieties are covalently bound to target proteins. The polymer has short half-life of less than 2 min (Wielcken *et al.*, 1983). It has also been seen that the half-life of polymers formed as a result of carcinogen treatment of cells is considerably shorter than the half-life of constitutive polymers (Wielcken *et al.*, 1983; Alvarez-Gonzalez *et al.*, 1985). The PAR reaction is schematically depicted in Fig. 3. A related process, mono-ADP-ribosylation, relates to bacterial signal transduction (Miwa and Sugimura, 1990; O'Farrell, 1995) and shall not be discussed in the thesis.

Three main enzymes are known to be involved in the PAR reactions.

Poly-ADP-ribose polymerase (PARP): The ubiquitous enzyme catalyses the sequential transfer of ADP-ribose units from NAD^+ donor to acceptor proteins. At least 5 different PARP have been identified (Jacobson and Jacobson, 1999). Although their functions are not yet well understood, their existence indicates a potential for considerable consumption of NAD^+ under specific conditions.

Poly-ADP-ribose glycohydrolase (PARG): This enzyme degrades poly-ADP-ribose units from the target proteins (Miwa *et al.*, 1981; Hatakeyama *et al.*, 1986).

ADP-ribosyl protein lyase: It catalyses removal of proximal ADP-ribose bound to a protein (Oka *et al.*, 1984).

1.3.1. Enzyme involved in poly ADP-ribose anabolism

PARP is the main anabolic enzymes of PAR reaction and has been very well characterized. It has been purified from various tissues like rat liver (Okayama *et al.*, 1977), calf thymus (Mandel *et al.*, 1977), human placenta (Shizuta *et al.* 1985; Yamanaka *et al.*, 1988), *etc.* It is highly unstable under biological conditions. PARP has been shown to contain 3 distinct functional domains. A substrate binding moiety of 54 kDa, a DNA-binding domain of 46 kDa and a site of automodification of 22 kDa polypeptide have been identified (Kameshita *et al.*, 1984; Shizuta *et al.*, 1985). Certain antigenic structures of the DNA-binding domain of PARP are highly conserved in various animal cells (Kameshita *et al.*, 1985). The reaction catalyzed by PARP is depicted below (Althaus and Richter, 1987):

$$\text{Acceptor} + \text{n NAD} \xrightarrow[\text{PARP}]{\text{DNA}} \text{Acceptor-(ADP-ribose)}_n + \text{n Nicotinamide} + \text{n } H^+$$

PARP is DNA dependent and is activated or stimulated by various DNA damaging agents including some carcinogens, radiation, Mg^+, polycations such as histones (Okayama *et al.*, 1977; Kristensen and Holtlund, 1978) or polyamines such as spermine, spermidine and putrescine (Kawamura *et al.*, 1981). This enzyme is also activated by different cellular factors or external interventions (Schneeweiss *et al.*, 1993; Sharan *et al.*, 1996; Homburg *et al.*, 2000; Zhang *et al.*, 2002). Histones are the main target proteins for PAR reactions (Sharan *et al.*, 1998a). PARP may also serve

as an efficient acceptor of ADP-ribose polymers and undergo automodifications (Yoshihara *et al.*, 1977) and eventually changes the kinetic properties of the enzyme (Kawaichi *et al.* 1981c). A lot of inhibitors have also been known that inhibit the activity of PARP. They have been of great help in the process of elucidating the biological significance of PAR reactions. It has been shown that substituted benzamides such as 3-aminobenzamide (3-AB) and 3-methoxybenzamide are potent physiological inhibitors of PARP at different concentrations (Purnell and Whish, 1980 a).

1.3.2. Enzymes involved in poly ADP-ribose catabolism

The main catabolic enzyme of PAR reaction is poly-ADP-ribose glycohydrolase (PARG) (Miwa *et al.*, 1981). It cleaves ribose-ribose bond of both linear and branched portions of poly-ADP-ribose by an exoglycosidic hydrolysis mode. It has been reported to be present in almost all the eukaryotes except in yeast (Tavassoli *et al.*, 1983; Werner *et al.*, 1984; Lautier *et al.*, 1993). Its role is antagonist to PARP (Lautier *et al.*, 1993; Sharan *et al.*, 1996). Three potential modes of action of PARG have been proposed exoglycosidic, endoglycosidic and combination of both (Braun *et al.*, 1994). Recently it has been demonstrated that presence of endoglycosidic activity of PARG could have a vital role in the involvement of PAR metabolism during DNA-repair and other cellular responses (Desnoyers *et al.*, 1995).

1.4. TARGET PROTEINS FOR PAR

A large number of proteins have been discovered which are targets of PAR reaction. The acceptors proteins for PAR include many nuclear proteins such as histones, A24 proteins, high-mobility-group proteins, low-mobility-group proteins, nuclear scaffold proteins, topoisomerases I and II, DNA polymerases α and β, terminal nucleotidyltransferase, DNA ligase I and II, RNA polymerase II, Ca^{2+}, Mg^{2+}-dependent endonucleases, and PARP itself (Miwa and Sugimura, 1990; Sharan *et al.*, 1998; Pariat and Sharan, 1998; Devi, 2001; Kun *et al.*, 2002). The activities of most of these enzymes are inhibited following their PAR (Boulikas, 1991). The covalent attachment of first ADP-ribose moiety occurs on arginine (Takenaka *et al.*, 1995), cysteine (Jacobson *et al.*, 1990), asparagines (Sekine *et al.*, 1989) and diphthamide, a modified histidine (Iglewski and Dewhurst, 1991). Free ADP-ribose can react non-enzymatically with lysine and cysteine (McDonald and Moss, 1993) residues.

1.5. CELLULAR DISTRIBUTION OF PAR

PAR was first discovered in liver nuclei (Chambon *et al.*, 1966). The natural occurrence of PAR activity has been shown in a large group of different phyla, classes and species. The presence of PAR activity appears to be almost ubiquitous among higher eukaryotes. From mammals, aves down to fungi, slime molds and algae. Man, bovine, mouse to *Tetrahymena pyriformis*, *Crypthecodinium cohnii*, *etc.* all display PAR activity (Althaus and Richter, 1987). Almost all tissues and cells have been shown to have PAR activity except mammalian erythrocytes, which loses its nuclei during erythropoiesis. PAR activity has also been shown in plants (Whitby *et al.*, 1979) and yeast (Hayaishi and Ueda, 1982) although the latter is still controversial. PAR activity has also been shown in many cell culture systems like human monocytes, HeLa cells, human kidney T1 cells, mouse L929 cells, mouse L51784

lymphoma cells, chick myoblasts, *Drosophila*, tobacco cells, *etc.* (Ferro and Olivera, 1982; Althaus and Richter, 1987; Schneeweiss *et al.*, 1995). These cultures have provided a lot of information on PAR and its biological functions and significances.

The extra nuclear existence of PAR activity was shown for the first time in ribosomes (Roberts *et al.*, 1975). Subsequent studies by different scientists have shown its existence in mitochondria (Kun *et al.*, 1975), various post mitochondrial fractions (Kun *et al.*, 1975), microsomal-ribosomal fraction of rat spermatogenic cells (Concha *et al.*, 1989), *etc.* It has been shown to bear the characteristic features of the nuclear PAR activity such as dependence on DNA, inhibition by PARP inhibitors, *etc.*, with some exceptions.

1.6. INVOLVEMENT OF PAR IN CELLULAR PROCESSES

A multitude of cellular functions and molecular events, including carcinogenesis, gene expression, replication, DNA damage and repair, *etc.* are known to have strong involvement of PAR (Boulikas, 1993; Masutani *et al.*, 1995; Schneeweiss *et al.*, 1995; Sharan, 1996; Sharan *et al.*, 1996; Böhm *et al.*, 1997; Saikia *et al.*, 1999; Chang *et al.*, 2002; Cinel *et al.*, 2002; Fiorillo *et al.*, 2002; Gonzalez *et al.*, 2002; Pacher *et al.*, 2002).

1.6.1. PAR and chromatin structure

All chromatin functions such as DNA repair, DNA replication and transcription, *etc.* are undoubtedly accompanied by local alteration of the chromatin organization. Researches, therefore, suggest that the PAR of nuclear proteins induces local alterations in the architecture of active chromatin domain (Althaus and Richter, 1987; Hough and Smulson, 1994; Saikia, 1996; Pariat, 1997; Devi, 2001).

The highly complex chromatin structure consists of repetitive units of proteins complexed with DNA that is packaged into several levels or orders of coiling. Each nucleosome, the subunits of chromatin, is composed of a core histone octamer and a stretches of 200 bp of DNA wrapped approximately twice around it (Boulikas, 1991; Luger *et al.*, 1997). Nucleosomes are organized into second-order structures by interactions between the 1-72 amino terminal segment of H1 and the core histones in neighboring nucleosomes. Such structures are described by the double-helical crossed-linker, the helical ribbon or the solenoid structure. A further coiling of the second-order chromatin structure may form third-order chromatin structures. This leads to condensed transcriptionally inactive heterochromatin. All levels of chromatin structures are organized into loops for a compact, coiled structure (Boulikas, 1991).

The evidence of PAR of chromosomal proteins opening up higher order chromatin structure *in vitro* was given by the works of Poirier *et al.* (1982) and Neidergang *et al.* (1985). It was shown that PAR directly affects the function of H1. This alters higher order conformational states of chromatin. The group of Poirier has shown that chromatin was unable to condense when histones (mainly H1) were poly-ADP-ribosylated using the endogenous polymerase (Poirier *et al.*, 1982; Aubin *et al.*, 1983). It has been further shown that although a very tiny fraction of H1 is poly-ADP-ribosylated (less than 5% of the total) it can cause drastic changes in the chromatin structure (Mathis and Althaus, 1987). Poly ADP-ribose has been shown to exert

significant influence in the interactions of DNA with histones H1, H3 and H4 (Wesierska-Gadek and Sauermann, 1988). Boulikas (1990) proposed that PAR of histone H2B, to H1, other core histones or to polymerase could bring about a transient dissociation of core histone octamer from DNA in nucleosome undergoing repair. It can also affect new nucleosome formation at replication fork (Boulikas, 1991). The lowering of PAR, particularly of H1 and some core histones, has been shown to be accompanied by relaxation of chromatin superstructure (Devi, 2001). Histone removal from DNA by binding to a molecule of poly-ADP-ribose might be mediated by the equal or higher affinity of H3 and H4 for poly-ADP-ribose than for DNA (Wesierska-Gadek and Sauermann, 1985). PAR might then be rapidly degraded by PARG. This allows the dissociation of the histones from the DNA at the damaged sites or at the replication fork. The PARP then could modify histones at the adjacent nucleosome and so on (Hough and Smulson, 1994). Due to high polymer turnover, a small fraction of modified H1 would be detected at any given time (Boulikas, 1991). The model elaborated above requires coordination and close association of PARP and PARG. The affinity of PARG for its substrate, poly-ADP-ribose attached to the polymerase, might be responsible for the association of two enzymes (Boulikas, 1991).

Many findings have shown that the PAR of H1 does not result in its dissociation from chromatin but causes chromatin relaxation (Poirier *et al.*, 1982; Aubin *et al.*, 1983; Neidergang *et al.*, 1985; de Murcia *et al.*, 1986). Induction of local condensation of chromatin by PAR has also been reported (Wong *et al.*, 1983 a). It was convincingly shown that hyper-ADP-ribosylation of H1 and H2B and PARP itself occurs. They are responsible for the altered conformational changes at the solenoidal level of chromatin (Althaus and Richter, 1987). The interaction of ADP-ribose polymers of automodified PARP *in vivo* with adjacent proteins, particularly histones, may induce alterations in chromatin conformation through noncovalent interactions with histones (Panzeter *et al.*, 1992; Jacobson and Pillus, 1999). It has also been shown that chromatin remodeling during embryogenesis involving changes at the attachment points of the chromatin loops or domains due to nuclear matrix. This may be controlled by PAR of topoisomerases, histones or matrix proteins (Boulikas, 1990). It was shown using isolated nuclei that PAR of one core histone in a nucleosome was enough to disrupt the interaction between histones H2B and H4 or H3 and H2A. This occurs only in the nucleosome associated with the nuclear matrix but not in bulk chromatin nucleosomes. When these findings were related to *in vivo* situation, it indicates certain things. The higher sensitivity of nuclear matrix nucleosomes to ADP-ribosylation might have consequences on the association between nuclear matrix proteins and nucleosomes. This might be the initial step in chromatin loop remodeling (Boulikas, 1987). This process might move a gene from an inactive into an active chromatin domain and *vice versa* (Boulikas, 1991). Chromatin replication, repair and transcription occur in association with the nuclear matrix (Boulikas, 1987; Hancock and Boulikas, 1982). External stimuli and radiation have been shown to influence PAR as well as the structural organization of chromosomal DNA (Schneeweiss *et al.*, 1995; Sharan *et al.*, 1998a).

1.6.2. PAR and DNA replication

The transformation and tumorigenesis processes are closely linked to the control of DNA replication and the entry of cells into mitosis (Boulikas, 1991). Several independent investigations have shown link between PAR and DNA replication. It has

been shown that there is increase in the level of PAR or the activity of PARP in S phase in synchronized cells (Kidwell and Mage, 1976; Berger *et al.*, 1987; Alkhatib *et al.*, 1987) and in G1 phase preceding DNA replication (Thibodeau *et al.*, 1989). The activity of PARP is higher in actively dividing cells in comparison with the quiescent cells (Gill, 1972; Stone and Shall, 1975). Mitogen-stimulated lymphocytes show higher PAR level compared with unstimulated lymphocytes (Lehmann *et al.*, 1974). Similar enhancement of PARP activity was seen in leukemic cell nuclei when compared with normal human lymphocytes (Burzio *et al.*, 1975). In another work by Anachkova *et al.* (1989) it was shown that isolation of a chromatin fraction enriched in replication chromatin shows a 2- to 3-fold enrichment in PAR level compared to the bulk chromatin. SV40 DNA replication was shown to be inhibited by poly-ADP-ribosylated diadenosine tetraphosphate (Ap4A). Both Ap4A and poly-ADP-ribose accumulates in the mammalian cells following stresses that are accompanied by DNA strand breaks. Therefore, poly-ADP ribosylated Ap4A could inhibit DNA replication, allowing DNA repair to occur (Baker *et al.*, 1987).

1.6.3. PAR and gene expression

Gene expression is a well-regulated and well-coordinated molecular process. Several genes are put on and off depending upon the need of the cell. Any abnormality in this process can lead to severe biological consequences. Often the transformation or tumorigenesis in a cell results due to over or under expression of specific genes and/or point mutation in an oncogene (Berns, 1999). Gene expression requires topological alterations in the DNA superhelical structure. This leads to relaxation of part of DNA and involves several proteins/factors. It has been shown that PAR reactions play an important role in the regulation of gene expression (Nagao *et al.*, 1991; Smulson *et al.*, 1995). It has been shown that topoisomerase I, upon poly-ADP-ribosylation in intact cells, result in its inactivation (Krupitza and Cerruti, 1989). This implies that the rate of transcription in several nuclear microenvironments might be controlled by PAR. PAR might be a mechanism for slowing down or shutting off transcription and replication. This may allow repair to occur in heavily damaged cells. However, under physiological conditions and in the absence of heavily damaged DNA, PAR of histones is proposed to act to facilitate transcription and replication (Boulkas, 1991). Thus, PAR of histones proteins might be involved in maintaining transcriptionally active chromatin domain (Boulikas, 1990; Boulikas *et al.*, 1990).

ADP-ribosyl groups on histone N-termini would remove positive charges. This would weaken histone-DNA interactions that might result in partial nucleosome unfolding. Open nucleosome structure seems to be present in the transcriptionally active chromatin (Boulikas, 1991). Huletsky *et al.* (1989) have shown opening up of nucleosome by PAR of H1. It has been shown that nicotinamide, an inhibitor of PARP, decreases the activity of RNA polymerases I, II, III in calf and human thyroid tissues (Kleiman de Pisarev *et al.*, 1987). Also, inhibition of transcription by α-amanitin or actinomycin D caused a reduction in PAR. PAR of HMG proteins were also shown to occur in intact cells might play a role in the regulation of gene expression (Tanuma *et al.*, 1983; Pariat, 1997; Pariat and Sharan, 2002). Indeed, HMG proteins are associated with transcriptional active genes (Weisbrod, 1982; Boulikas, 1987; Owen-Huges and Worleman, 1994). Large T antigen, interacting with the origin of replication of SV40, is also poly-ADP-ribosylated in intact monkey cells.

This might regulate viral gene expression and initiation of virus replication (Goldman *et al.*, 1981; Baksi *et al.*, 1987).

Fakan *et al.* (1988) have shown that automodified PARP was preferentially associated with transcriptionally active chromatin at micro-nuclear environments harboring nascent ribonucleoprotein fibrils. Acetylated histones are known to be enriched in transcriptionally active chromatin. They are also the targets for PAR (Boulikas, 1990; Boulikas *et al.*, 1990). Logically, therefore, both acetylation and PAR of histones should essentially cause similar structural changes in chromatin. Indeed this has been shown (Böhm *et al.*, 1997). These evidences and other data suggest that histone PAR may regulate chromosome superstructure and gene expression (Boulikas, 1991; Böhm *et al.*, 1997).

1.6.4. PAR and cell differentiation

The involvement of PAR in cellular differentiation has been observed in different studies strengthening the concept that PAR is required for the initiation of differentiation (Ikai *et al.*, 1980b; Nagao *et al.*, 1991; Smulson *et al.*, 1995; Taylor and Williams, 1998). Most of the studies on involvement of PAR in cellular differentiation have made use of inhibitors of PARP activity. The PARP activity appears to be regulated at posttranslational level (Herzog *et al.*, 1989; Bhatia *et al.*, 1990; Sharan *et al.*, 1996; 1998b). Caplan and Rosenberg (1975) reported first evidence of the involvement of PAR in differentiation of mesodermal cells of embryonic chick limbs. Nicotinamide and benzamide compounds, inhibitors of PARP, were used in the study. PARP activity was shown to decrease during granulocyte differentiation of the human promyelocytic cell line HL-60 (Kanai *et al.*, 1982). The level of PARP mRNA also decreased during retinoic acid-induced granulocytic differentiation of human HL-60 cells (Suzuki *et al.*, 1987) and during nerve growth factor induced neurite outgrowth in rat pheochromocytoma cells (Taniguchi *et al.*, 1988). During differentiation of the human promyelotic leukemic cell line HL-60 with DMSO or retinoic acid along the myelotic lineage (Farzaneh *et al.*, 1987) or of primary avian myoblast, murine erythroleukemia cells, the normal human granulocyte-macrophage progenitor CFU-gm cells and during aging (Farzaneh *et al.*, 1982; Terada *et al.*, 1978; Johnstone and Williams, 1982; Jackowski and Kun, 1981) the number of DNA single-stranded breaks as well as level of PAR were found to increase.

1.6.5. PAR and chromosome stability

Breakage and reunion of DNA strands are related to fundamental functions associated with chromatin. It is likely to be involved in sister chromatid exchange (SCE), DNA repair, gene amplification and gene arrangements, including integration and deletion of genes (Miwa and Sugimura, 1990). The inhibitors of PAR seem to have profound effects on chromosome stability (Wang *et al.*, 1997). Many mutagens and carcinogens cause SCE involving breakage and reunion of homologous chromatids of DNA (Miwa and Sugimura, 1990). It has been shown that inhibitors of PARP, such as nicotinamide and others, induce SCE. These inhibitors also delay cell cycle progression (Zwanenburg and Natarajan, 1985). The extend of induction of SCE correlates well with the extend of *in vitro* inhibition of PARP activity (Oikawa *et al.*, 1980). Inhibitors of PARP did not, however, induce chromosomal aberrations or point

mutations in Chinese hamster ovary cells at the hypoxanthine-guanine phosphoribosyltransferase locus. In combination with alkylating agents or with UV or ionizing radiation, it did increase the frequency of both chromosomal aberrations and mutations at this locus (Natarajan *et al.*, 1983; Natarajan *et al.*, 1982; Schwartz *et al.*, 1985). Therefore, much of the works in this line does provide an indication of a correlation between SCE and PAR reaction. It has been reported that mutation in human PARP gene affects cell recovery, apoptosis and SCE following DNA damage (Schreider *et al.*, 1995). Inhibition of PAR has been reported to induce DNA hypermethylation (Zardo *et al.*, 2002). It has also 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).

1.6.6. PAR in DNA damage and repair

Several agents can interact with the DNA. The interactions cause various types of damage and bring about alterations in the DNA structure. This results in changes in the functionality of the DNA. Eventually it leads to cellular changes that might be detrimental to cell survival. There are strong evidences that PAR may regulate the repair process in DNA damage by inducing PARP. It has been shown that a UV-sensitive tumorigenic murine lymphoma cell line undergoes spontaneous conversion into resistant non-tumorigenic subline. This change coincides with a 3-fold increase in the level of PAR (Kleczkowska *et al.*, 1990). Carcinogenic compounds as well as inhibitors of PARP induce sister chromatid exchanges (Utakoji *et al.*, 1979). This implies that PAR plays an important role in preventing sister chromatid exchange-induced cancer (Boulikas, 1991). In fact DNA rearrangements have been found in cancer cells that are probably induced by an excessive amount of unrepaired DNA strand breaks (Yunis, 1983). It is established that chromatin structure plays an important intermediate role in repair processes. This was shown in an experiment in which damage of cells with carcinogens exhibit a 20 to 30-fold increase in H1 poly-ADP-ribosylation (Thi Man and Shall, 1982; Kreimeyer *et al.*, 1984). PAR has been suggested to regulate DNA excision repair, especially at the step of joining DNA breaks (Althaus and Richter, 1987; Miwa and Sugimura, 1990; Shall, 1994). The induction of DNA strand breaks by alkylating agents or radiation is associated with a decrease in the cellular NAD^+ level. There is a concomitant increase in PARP activity in nuclei and permeable cell during this time (Berger *et al.*, 1981; Skidmore *et al.*, 1979). During the phase of rejoining DNA strand breaks, a considerable amount of NAD^+ is consumed (Benjamin and Gill, 1980). Inhibitors of PARP can prevent the decrease in NAD^+ and also impair the rejoining of DNA (Durkacz *et al.*, 1980). It has been suggested that PARP is not necessary repair enzyme, but is able to stimulate the excision repair pathway (Lindahl *et al.*, 1995). This is in contrast to result obtained from the histone-shuttle model (Althaus, 1992). A model of a histone independent participation of PARP in the excision repair pathway has also been proposed (Satoh and Lindahl, 1992; Schreider *et al.*, 2002). PARP gene knocked-out mice showed no drastic reduction in their DNA repair capability (Wang *et al.*, 1997). This implies that PARP is not directly participating in DNA excision repair. Increase in unscheduled DNA synthesis by inhibitors of PARP after exposure to DNA-damaging agents has also been reported (Sims *et al.*, 1982). Thus, the exact mechanism of involvement of PAR in repair is not known. However, PAR continues to play significant role in the

regulation of DNA repair processes, particularly the base excision repair process (Dantzer *et al.*, 1999).

1.7. PAR AND CARCINOGENESIS

PAR has been shown to be associated with various cellular processes leading to carcinogenesis. Correlation between the cellular PAR and different cellular and molecular events has been observed in our laboratory and elsewhere (Boulikas, 1993; Sharan, 1996; Sharan *et al.*, 1996; Pariat, 1997; Saikia *et al.*, 1999; Devi, 2001). The earliest report of role of PAR in cell transformation came from the fact that malignant or transformed cells *in vitro* had higher PARP activity than the normal or untransformed cells (Miwa and Sugimura, 1990). Gene expression, replication, DNA damages and repair, cell differentiation, chromosome instability, chromatin structure, *etc* are some cellular and molecular events where strong involvement of PAR has been reported (de Murcia *et al.*, 1988; Boulikas, 1993; Sharan *et al.*, 1996; 1998a; Saikia *et al.*, 1999; Schneeweiss *et al.*, 2000). Most of these processes are directly or indirectly related to carcinogenesis. Relationship between PAR and carcinogenesis can also be established by the facts that the repair of DNA strand breaks shows an absolute dependence upon the rapid synthesis and degradation of poly ADP-ribose (Boulikas, 1993). Also that treatment of culture cells with tumor promoters have shown enhancement in the activity of PARP without the appearance of DNA strand breaks (Miwa and Sugimura, 1990). Accumulation of unrepaired DNA strand breaks in the cell accelerating gene rearrangements; deletions, insertions and amplification can lead to cancer development (Boulikas, 1991). These findings also point out indirectly to a possible correlation between PAR, chromatin and carcinogenesis. A direct relationship between the relaxation of poly-nucleosome structure and PAR has been shown (de Murcia *et al.*, 1988; Boulikas, 1993; Sharan *et al.*, 1996; 1998a; Saikia *et al.*, 1999; Schneeweiss *et al.*, 2000; Devi, 2001). It has been shown that treatment with chemical carcinogens inhibit PAR of different chromosomal proteins (Saikia, 1996; Pariat, 1997; Devi, 2001). It has also been suggested that lowering of PAR is a hallmark during the initial phase of carcinogenesis and tumorigenesis (Devi, 2001). Limited results from experiments with cells in culture and with laboratory animals show that inhibition of PAR alters course of cellular transformation and tumorigenicity brought about by a number of carcinogens. Cancer is believed to have three distinct stages (Pitot, 1986) and PAR have been shown to be involved in all three stages, namely initiation, promotion and progression (Miwa and Sugimura, 1990). Involvement of PAR has been seen in a number of diseases in experimental animals and human, including some autoimmune diseases, although no direct relationship between cancer-prone hereditary diseases and PAR is known so far (Miwa and Sugimura, 1990). Antibodies against poly-ADP-ribose and PARP were seen in patients with systemic lupus erythematosus, rheumatic paresthesia, myalgia, induced-diabetes mellitus, *etc.* (Miwa and Sugimura, 1990). It has been proposed that induction of changes in the DNA structure might lead to altered gene expression and, hence, indirect initiation of carcinogenic activity cannot be ruled out completely. Of all known inducers of carcinogenic transformation, chemical carcinogens are of special interest from the human safety viewpoint because of persistent increase in its availability to human population in the present modern world (Sikora, 1999).

1.8. CHEMICAL CARCINOGENESIS

There are numerous chemicals or compounds that has been attributed to cancer and can be widely grouped into the following sub-classes (OSHA, 1991):

Sub-class I:
Substances or group of substances and medical treatments that are known to be carcinogenic. Some of them are aflatoxins, azathioprine, analygesic mixtures containing phenacetin, arsenic and certain arsenic compounds, benzene, conjugated estrogens, cyclophosphamide, diethylstilbesterol, erionite, melphalan, methoxsalen with ultraviolet A Therapy (PUVA) mustard gas, thorium dioxide, vinyl chloride, *etc*.

Sub-class II:
Substances or group of substances and medical treatments that may reasonably be anticipated to be carcinogenic. Among these are acetaldehyde, acrylamide, acrylonitrile, 2-aminoanthraquinone, beryllium and certain berylliun compounds, carbon tetrachloride, chlorinated paraffins (C12, 60% chlorine), C. I. Basic Red 9 monohydochloride, cisplatin, DDT, dimethylcarbamoyl chloride, direct black 38, estrogens (not conjugated-estradiol-17, estrone, ethinylestradiol, mestranol), lead acetate and lead phosphate, nitrofen, nitrogen mustard hydrochloride, a large number of N-nitroso compounds such as N-Nitrosodiethanolamine, DMN, DEN, N-nitroso-N-methylurea, *etc*., benzo[a]pyrene, saccharin, silica, crystalline (respirable)-quartz, thiourea, urethane, *etc*.

Sub-class III:
Occupational exposures associated with technological processes that are known to be carcinogenic. It includes coke oven emissions, soots, tar and mineral oils, *etc*.

This list of chemical carcinogens is in no sense complete and the items that are added to it is on a rise with the progress of time.

1.9. DIMETHYLNITROSAMINE

Dimethylnitrosamine (DMN) is a member of class of N-nitroso compounds. N-nitroso compounds are considered separately because they induce chromosome breaks and aberrations and some of them are extremely potent point mutagens in cellular systems (Marquardt *et al*., 1964; Kihlman, 1966; Magee and Barnes, 1967).

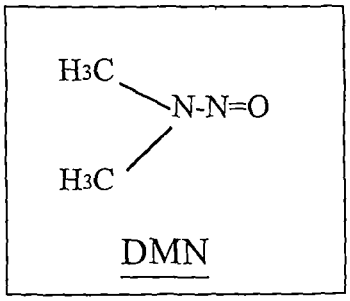


Figure 4: Chemical structure of dimethylnitrosamine (DMN).

Thus, it is a potent carcinogen and cause of considerable concern from human safety viewpoint. DMN may be found in food and many biological components contain nitrogen that can give rise to this compound after specific chemical reaction. Low concentrations of N-nitroso compounds have been detected in air, water, food mainly in nitrite-treated meat products and certain fish products. The *in vivo* formation of N-nitroso compounds from nitrates or nitrites and amines or amides have been demonstrated in experimental animals and in man (WHO, 1978). The chemical structure of DMN is given in Fig. 4. Some of its important chemical properties are given in the table below.

Table: Chemical properties of DMN

Character	Property
Empirical formula	C_2H_6-N_2O
Molecular weight	74.08
Percentage composition	C, 32.42 %; H, 8.16 %; N, 37.82 %; O, 21.60 %
Form and color	Yellowish liquid
Boiling point	151-153 °C
Density	1.0048
Refractive index	1.4363
Solubility	Very soluble in water, alcohol and ether
Lethal dose (i. p. in rats)	32.2 - 40.0 mg kg^{-1} body weight

DMN is a stable compound, which can interact with DNA only after enzymatic activation (removal of one alkyl group). It is a known hepatocarcinogen (Magee and Barnes, 1956; Pariat and Sharan, 1995; Devi, 2001). It is also known to induce mutations in *Drosophila*, whereas they are not effective in bacteria, yeast, or *Neurospora*, unless they are activated either chemically or enzymatically in the host mediated assay (Magee and Barnes, 1956).

DMN is a well-defined animal nitrosamine pro-mutagen (Lijinsky, 1976). Nitrosamines have been proven to be complete carcinogens and can acts as initiators and promoters (Wigley *et al.*, 1985). This is supported by the findings that these compounds cause carcinogenesis in mice and result in alteration in the PAR activity (Pariat, 1997; Pariat and Sharan, 1998; Devi, 2001). The activation of this nitroso compound by mammalian S-9 requires NADPH and cytochrome P-448 and P-450 systems (Guttenplan *et al.*, 1976). The carcinogenic activity is dependent upon the oxidative dealkylation carried out by microsomal mixed-function oxidase in the endoplasmic reticulum of susceptible tissues. Diazomethane may be formed, and the ultimate carcinogen is the alkyl carbonium ion, which reacts with nucleic acid (Guttenplan *et al.*, 1976). The first report of the direct mutagenic activity of this compound *in vivo* was demonstrated in a host-mediated assay (Gabridge and Legator, 1969). The mutation frequency from five separate trials with 0.1 ml of 10 % DMN was 1.3 x 10^{-6}; the lowest concentration giving a positive response was 20-50 mg/kg. Occurrence of toxic liver injury by DMN in man and rats and carcinogenesis by the same compounds in rats has also been reported (Magee and Barnes, 1956). DMN is also a carcinogen in plants. Veleminsky and Gichner (1968) treated seeds of *A. thaliana* with several nitrosamines and discovered that DMN (490 mM, 24 h) induced recessive chlorophyll mutations. They suggested that *Arabidopsis* seeds contained specific enzymes that decomposed the nitrosamines into mutagenic agents. After this discovery, it was reported that the dose-response curves of *Arabidopsis* were roughly parabolic, with increased mutation frequency and killing at the lower doses of DMN

(up to 800 mM) (Veleminsky and Gichner, 1971). It has been reported that DMN induces chromosomal aberrations in barley (Sutzhi and Kaul, 1972). DMN has also been shown to induce somatic mosaicism in *Glycine max*. A maximum frequency of somatic crossing-over was observed when seeds were treated with the concentration of 60 ppm. The lowest concentration of DMN that produce leaf spots was 1.25 ppm (Arenaz and Vig, 1978a; 1978b).

The maximum effect induced by DMN indicates a saturation limit of the system that converts the nitrosamine into a mutagen. It has been shown that the principle product of methylation of DNA by DMN is 7-methylguanine, followed by 3-methlyadenine (Pegg and Hui, 1978). However DMN also induces appreciable amounts of O^6-methylguanine. There is substantial body of experimental data showing that the methylating species derived from DMN react with DNA to produce 7-methylguanine and O^6-methylguanine in the relative proportion of about 9:1 (Lawley, 1974; Pegg and Hui, 1978; Pegg, 1977). Thus, the ratio of O^6- to 7-methylguanine ranged from 0.10-0.125 for DMN (Lawley, 1974; Pegg and Hui, 1978; Pegg and Nicoll, 1976; Pegg, 1977; Kleihues and Margison, 1976; Margison and O'Connor, 1973). This value refer to the initial proportion of the methylation at the O^6-position of guanine to that at the N-7 position, since it has been shown that both alkylated guanines can have the same or different persistence, depending on the dose and repair abilities of the alkylated cells (Lawley, 1974; Swenson and Lawley, 1978; Frei *et al.*, 1978; Rajewsky and Goth, 1976). Phosphodiesters are also formed in relatively large amounts in the reaction of DMN with DNA.

It is, thus, clear that DMN is a potent carcinogen in various groups of organisms, including man. Its carcinogenic effect is through its activation in the host organism by microsomal mixed-function oxidase generating electrophilic reactive carbonium ions that can interact and cause alteration of DNA structure and, thereby, its functionality, leading to the initiation of carcinogenesis. DMN and other N-nitroso compounds can be readily formed by the interaction of secondary or tertiary amino compounds with nitrous acid or other nitrosating agents (Lijinsky, 1976). Nitroso compounds can be formed easily in the acid of stomach from ingested nitrite and amino compounds (Mirvish, 1995). They can be formed but less readily in nonacidic conditions when catalysts or other suitable agents are present (Keefer and Roller, 1973) and they have been reported at low concentrations in food processed with nitrite. They must, therefore, be considered environment carcinogens and mutagens of some importance (Lijinsky, 1976).

1.10. AQUEOUS EXTRACT OF BETEL NUT (AEBN)

The betel or areca palm (*Areca cetechu* L.) is indigenous plant species of South Asia. It grows in most parts of South, South-East Asia and Pacific Ocean islands (Majumdar *et al.*, 1979). In these regions, the nuts of the palm, commonly called betel nut, are widely consumed either alone or as a constituent of a quid, along with betel leaves (*Piper betel*), slaked lime and chewing tobacco. Different ethnic groups, however, consume it in different forms. Mastication of the betel nuts in religious and social ceremonies is a part of old custom and tradition. It is estimated that betel nut is cultivated in about 4132 ha in Malaysia, 79521 ha in Bangladesh, 19659 ha in Sri Lanka and 174300 ha in India (Nayar, 1974). In India, it is commercially cultivated in many parts, mainly in the eastern and north-eastern regions.

Reference of areca nut or betel nut can be found in ancient Indian texts. There is reference to areca nut in *Vagbhata* (4th century), possibly the earliest reference of areca nut in any text (Majumdar *et al.*, 1979). In this text the role of areca nut in curing various diseases such as leucoderma, leprosy, cough, fits, worms, anaemia, obesity, nasal ulcer, *etc.* are mentioned. The appetizing and stimulating properties of areca nut are mentioned in *Bhavamista*, 13th century (Majumdar *et al.*, 1979). There is also a description of seven varieties of white betel nut (Watt, 1889). The powdered seed of betel nut acts as antihelmintic drug for dogs (Watt, 1889). Arecoline is the main and physiologically most active alkaloid present in betel nut. But other alkaloids are also found (Sharan, 1996). Arecoline is reported to cause a fall in blood pressure, constriction of pupil (Nadkarni, 1908) and was also used in treatment of hookworm infection in the past (Chopra, 1933). The tincture of betel nut can be used in curing bleeding gums, lumbargo and sore lips (Majumdar *et al.*, 1979). Roasted areca nut was also reported to be helpful in dysentery (Desai, 1927).

Earliest report on chemical content of betel nut was recorded by Bombelon (Watt, 1889). Since then a lot of independent works have established the chemical composition of betel nut. It contains alkaloids such as arecaine, arecaidine, arecoline, guvacoline, guvacine and choline (Sharan, 1996). Other main constituents of betel nut are polyphenols, tannins, free amino acids, fats, free fatty acids, polysaccharides, crude fibre, mineral salts, water-soluble and water-insoluble ash, *etc.* (Mathew *et al.*, 1964; Grasser, 1929; Nanda and Kapoor, 1971; Sharan, 1996). Pharmacological studies have shown that various components of betel nuts, particularly the alkaloids, affect various metabolic functions. Arecoline have been shown to be effective in the cure of different helminthes (Majumdar *et al.*, 1979) and *Schistosoma mansoni* infection. Some alkaloids of betel nut such as arecoline, muscarine and pilocarpine are also reported to stimulate autonomic effector cells, which are acted upon by cholinergic post-ganglionic nerve impulses. They show the central stimulant action and result in bradycardia, hypotension, increased in intestinal tone, salivation and sweating (Leslie, 1965). Arecoline, the predominant alkaloid of betel nut, has also been shown to mediate cardio-vascular effects through cholinergic system, central nervous system (Majumdar *et al.*, 1979) and influence urine and electrolyte excretion perhaps by increasing the Na, K and osmolarity of urine without altering the urine volume (Avurunin and Carter, 1970). It has been shown that arecoline penetrate the blood brain barrier like muscarinic drugs (Olds and Domino, 1969). At low dose arecoline has been shown to increase motor activity, water and food consumption, and food reinforcement (Majumdar *et al.*, 1979).

Several studies have implicated the habit of chewing betel quid with high risk of cancer of oral cavity and upper digestive tract (Stich *et al.*, 1983; IARC, 1985; Sharan, 1996). Cancer study in the several countries where the consumption is rampant supports this point (Atkinson *et al.*, 1964; Cooke, 1969; Jussawalla and Deshpande, 1971; Wahi, 1976; Hirayama, 1979; Henderson and Aiken, 1979; Jussawalla, 1981; Sanghvi, 1981; Stich *et al.*, 1983; IARC, 1985; Wary and Sharan, 1988; Rao and Das, 1989; Sharan, 1996). Therefore, there has been growing concern about the ill effects of excessive betel nut consumption to human health (Sharan, 1996). It was demonstrated that different varieties of the preparations of areca nut differs in their carcinogenic potential (Rao and Das, 1989). In this study it was shown that preparations of ripe and unprocessed areca nuts yielded tumor in mice. However,

treatment with processed nuts did not produce tumor. This has been attributed to the loss of different chemical constituents and inactivation of some alkaloids in the processed nuts, thereby, reducing its carcinogenic potential. The carcinogenicity of betel nut has been mainly attributed to its alkaloid, arecoline but polyphenols/tannins also contribute to it (Rao and Das, 1989; Wary and Sharan, 1988; Sharan and Wary, 1992; Sharan, 1996). In addition, it has also been shown that different extract of betel nuts have different carcinogenic potential (Wary and Sharan, 1988; Sharan and Wary, 1992; Balachandran and Sharan, 1995). Components of betel nut such as arecoline and arecaidine have been reported to cause induction of cell transformation (Ashby *et al.*, 1979; Saikia *et al.*, 1998; Saikia *et al.*, 1999). Arecoline has also been shown to induce papilloma of the esophagus and carcinoma of the glandular stomach in hamsters (Dunham *et al*, 1974; 1975). It has been proposed that the alkaloids of areca nut influence the process of carcinogenicity by disturbing immuno-surveillance (Rao and Das, 1989). It was indeed observed that arecoline administration resulted in the suppression of immune responses in mice (Shabuddin *et al.*, 1980; Selvan, 1988).

Aqueous extract of betel nut (AEBN) has been shown to induce strand breaks in DNA of mouse kidney cells *in vitro* (Wary and Sharan, 1988; Sharan and Wary, 1992). It has also been found that AEBN increased the rate of cell division and caused cell toxicity at different doses (Wary and Sharan, 1988). The genotoxic potential of AEBN and arecoline have also been shown to cause sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells (Dave *et al.*, 1992). In this study the DNA damage appeared to be dose dependent and low concentration and prolonged exposure showed maximum damage to the DNA. Both the AEBN and arecoline acted as potent inducers of sister chromatid exchange. Thus, AEBN and arecoline have the potential to exhibit positive genotoxic effects. However, AEBN exhibited higher genotoxic effect than arecoline (Dave *et al.*, 1992). This implies that apart from arecoline, there are other water-soluble alkaloids that have the potential to exert genotoxic effects (Sharan, 1996). Arecaidine, which accounts for a small fraction of the areca nut alkaloid, also exhibits mutagenic activity (Shirname *et al.*, 1983; 1984). Induction of unscheduled DNA synthesis was induced in human cells following exposure to arecoline and different extracts of betel nut *in vitro* also confirms it (Sharan and Wary, 1992). In another study by Pariat and Sharan (1998) it has been shown that PAR of HMG proteins of liver cells was modified after AEBN treatment. HMG proteins are known to play a key role in the organization and function of chromatin (Schneeweiss *et al.*, 2000). Therefore, it has been postulated that changes in HMG proteins might alter the chromatin structure resulting in altered gene expression and might lead to the initiation of carcinogenesis. It was subsequently shown that upon AEBN treatment, there was progressive lowering of PAR of HMG proteins affecting its association with chromatin. This could lead to relaxation of chromatin, resulting in increase in the accessibility of carcinogens to DNA. This may have resulted in induction of mutation (Pariat and Sharan, 2002). These changes were believed to favor initiation of cancer induced by AEBN (Pariat and Sharan, 2002). Exposure to arecoline, the main alkaloid of betel nut, has been shown to reduce PAR in spleen cells (Saikia *et al.*, 1999).

1.11. 3-AMINOBENZAMIDE (3-AB) AND ITS ROLE IN CARCINOGENESIS

The use of the inhibitors of PAR has shed new light on the role of PAR in the cellular metabolism especially in carcinogenesis. The inhibitors are mainly for the PARP, the

anabolic enzyme in the PAR reactions (Rankin *et al.*, 1989). Substituted benzamides are the prime inhibitors of PAR, specifically PARP (Purnell and Whish, 1980a). The substituted benzamides include 3-acetoamidobenzamide, 2-aminobenzamide, 3-aminobenzamide, 4-aminobenzamide, 3-methoxybenzamide, *etc.* They effectively inhibit the PARP at different concentrations (Christine and Bernard, 1987; Devi, 2001). In addition several other chemicals are also known to inhibit the PARP activity. Some of them are nicotinamide and its derivatives such as 5-methylnicotinamide, 6-aminonicotinamide, diadenosine 5′, 5‴-P^1, P^4-tetraphosphate (Ap_4A), thymidine, theophyllin, cyclic AMP, caffeine, hypoxanthine, guanine, 6-mercaptopurine, proflavin, pyrazine, *etc.* (Steinhagen *et al.*, 2002). At different concentrations and reaction conditions, all these and others are known to inhibit PARP activity.

Among the substituted benzamides, 3-aminobenzamide is very significant because it can inhibit PARP activity under physiological conditions and has been shown to act effectively *in vivo* (Purnell and Whish, 1980a; Devi, 2001). It is effective at a low concentration (about 2 mM). 3-AB have been shown to be involved in almost all processes that involves PAR reaction, such as cellular differentiation, DNA damage and repair, DNA replication, gene expression, chromatin structure, genomic instability, tumorigenesis or carcinogenesis, *etc.* (Boulikas, 1991). 3-AB has been shown to induce SCE involving breakage and reunion of DNA strands and, thereby, altering the chromatin structure. This is related to inhibition of PARP (Miwa and Sugimura, 1990). It has also been shown that MNNG-induced amplification of integrated simian virus 40 (SV40) DNA is enhanced by 3-AB in a Chinese hamster cell line, CO 60 (Bürkle *et al.*, 1987). Inhibition of mammalian DNA replication by 3-AB has been shown (Miwa and Sugimura, 1990) and as we have discussed earlier, PAR is seen to be involved in the replication process. In another experiment it was shown that the degree of chromosomal aberrations was high in lymphocytes from Down syndrome patients when compared with the normal lymphocyte on X-ray irradiation in presence of 3-AB (MacLaren *et al.*, 1989). In human melanoma CRL 1424 cells, 3-AB have been shown to inhibit PARP activity resulting in the accumulation of 10 kb unligated DNA from the replication fork. This was interpreted as ligation required PARP activity (Lönn and Lönn, 1985). Inhibition of PARP by 3-AB or other inhibitors has been shown to increase cytotoxicity and mutagenicity of DNA-damaging agents, induce sister chromatid exchanges, chromosomal aberrations and increase carcinogen-induced unscheduled DNA synthesis (Boulikas, 1991). 3-AB in combination with other potent carcinogens have also been shown to increase transformation frequency (Lubet *et al.*, 1986).

Potentiation of cytotoxicity, increase in transformation frequency, blockage of cell division, inhibition of PARP activity, failure of ligation, alteration in the duration of cell cycle, increase in DNA breaks, increase in the cytogenic damage, increase in cell kill, reduction in nucleotide pools and cell death, *etc.* are some of the effects that are seen when 3-AB works in combination with other carcinogens in various cell type or systems (Morgan and Cleaver, 1983; Boulikas, 1991).

AIMS AND OBJECTIVES

In the backdrop of description detailed above, this piece of work embodied in this thesis was designed to explore and extend ways and means to make cancer detection

possible with applied potentials to implement it for screening human population, particularly in developing or under-developed countries like India. In this, biochemical characterization of the genome has been chosen for the reasons detailed earlier. The choice was also made due obvious ease in its application in countries like India. The biological end-point chosen for the investigation was status of poly ADP-ribosylation (PAR) of proteins following exposure of mice to either a specific carcinogen, dimethylnitrosamine (DMN), or a general, naturally occurring carcinogen, aqueous extract of betel nut (AEBN). An inhibitor of PAR, 3-aminobenzamide (3-AB), was also used in this investigation in combination with the carcinogens to further the insight. The following targets were set for the work:

1. To determine correlation between the levels of PAR of total cellular proteins in mouse tissues exposed to DMN and AEBN *in vivo* and different period of time within initiation stage of cellular transformation.
2. To determine correlation between the levels of PAR of total cellular proteins in mouse blood lymphocytes exposed to DMN and AEBN *in vivo* and different period of time within at the initiation stage of cellular transformation.
3. To determine the correlation between the levels of PAR of chromosomal proteins, histones, mouse tissues and blood lymphocytes exposed to DMN and AEBN and different period of time within initiation stage of cellular transformation.
4. To monitor effects of 3-AB in combination with DMN and AEBN on the same parameters listed above.
5. To analyze characteristics (e.g. structural alterations, gene activation, *etc.*) of genomic DNA (total chromosomes) at different period of time within initiation stage of cellular transformation using agarose gel electrophoresis (AGE) and pulse field gel electrophoresis (PFGE).
6. To explore the possible use of PAR as a cancer biomarker for screening of population.

Chapter 2

MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

All chemicals used were of reagent grade. Double-distilled water (H_2O) was used for the preparations of buffers and reagents. Barbituric acid, urea, sodium dodecyl sulphate (SDS), tris (hydroxymethyl) methylamine, glycine, sodium chloride (NaCl), non-fat dry milk, bromophenol blue, trichloroacetic acid (TCA), diphenylamine, sulphuric acid (H_2SO_4), sucrose, orcinol, hydrochloric acid (HCl), ferric chloride ($FeCl_3$), bovine serum albumin (BSA), Phosphoric acid, glacial Acetic acid, coomassie brilliant blue G-250, ethanol, methanol, xylene, ethylenediamine tetraacetic acid (EDTA), heparin, potassium phosphate, calcium chloride ($CaCl_2$), dithiothretol (DTT), RNA (type III from Bakers yeast), phenolphthalein, ammonia solution (NH_3), phenol, chloroform, isoamyl alcohol, ammonium acetate, agarose (low EEO), protein molecular weight markers, yeast artificial chromosome (PFGE markers), betel nuts, *etc.* were obtained locally. Polyvinylidene difluoride (PVDF) membrane and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets were obtained from Boehringer Mannheim, Germany. BCIP-NBT reagent from Bangalore Genei, India was also used in some experiments. Polyoxyethylene-sorbitan monolaurate (tween-20), acrylamide, bis-acrylamide, N, N, N′, N′-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), nitrocellulose membrane, β-mercaptoethanol, sephadex G-25, phosphate buffered saline (PBS) ready-to-use pouches, DNA (salmon testis), complete Freund's adjuvant, incomplete Freund's adjuvant, goat anti-rabbit antibodies conjugated with alkaline phosphatase, triton X-100, dimethylnitrosamine (DMN), 3-aminobenzamide (3-AB), magnesium chloride ($MgCl_2$), proteinase K, DNase I, *etc.* were procured from Sigma Chemical Co., USA. Goat anti-rabbit antibody-alkaline phosphatase conjugate was also obtained from Bangalore Genei, India. Silver staining reagents was obtained from Bio-Rad, USA. Rotring ink from Riepe KG, Germany was used. RPMI-1640 from Hyclone (USA), Biocoll separating solution from Biochrom KG (Germany), Ethidium bromide from Merck (Germany), was employed in the work.

2.2. INSTRUMENTS

Various makes and models of instruments were used in conduct of experiments and for the analyses of the results and data. Balances (Sartorius & Mettler); Varifuge 20 RS centrifuge (Heraeus); rotary vacuum pump, magnetic stirrers, ultra cryostat water bath shakers, tissue homogenizer, cyclomixers, quick freezer, ovens, Spinwin microcentrifuge (all from Remi Instruments, India); Eppendorf microcentrifuge (Ependorf); Lyolab 3000 freeze dryer with rotary pump (Heto-Holten, Denmark); ultrafreeze (Heto-Holten, Denmark); F90 compact electronic icematic® Castel MAC SpA (Italy), microscope (Carl Zeiss JENA 30-G0603); mini gel electrophoresis system, Mupid (Japan); Bio-dot®SF microfiltration apparatus, Mini-PROTEAN® II electrophoresis cell, Mini trans-blot® electrophoretic transfer cell, electrophoresis power supply 200/2.0, mini-transilluminator, gel dryer with vacuum pump and vapor trap, imaging densitometer GS-690 with molecular analyst PC software 1.5, (all from Bio-Rad, USA); gel electrophoresis apparatus GNA-100 (Pharmacia); electrophoresis power supply, 0-500V/0-500 mA, rocker-100 (both from Bangalore Genei, India); DU-60 spectrophotometer (Beckman); digital zoom camera DC120 with 1D image analysis software (Kodak digital science™) attached to Zenith PC LR4Dc; gene

navigator® system pulse field gel electrophoresis system (Pharmacia biotech, Sweden), *etc.* were equipments used throughout the work.

2.3. EXPERIMENTAL ANIMALS

Swiss albino mice (Balb/c) were used for the experiments. The mice were inbred and maintained in hygienic conditions in the animal room in different cages. The animal room was maintained at a temperature of 22-23 °C. The mice were fed with standard mice feed (Pranav Agro Industries Ltd., Delhi) and water *ad labitum.* Same age group (6-8 weeks), weighing 20-25 g, male mice were used for the experiments. Russian Chinchilla strain rabbit was used to raise polyclonal antibodies against ADP-ribose polymers. The rabbit was purchased from Pasteur Institute, Shillong, India.

2.4. CARCINOGEN PREPARATION AND TREATMENT PROTOCOLS

N-dimethylnitrosamine (DMN), a hepatocarcinogen, was used in this study (Magee and Barnes, 1967). DMN was administered at a dose rate was 10 mg kg^{-1} body weight in drinking water in a chronic oral administration protocol (DMN group) over a period of 4 weeks. Aqueous extract of betel nut (AEBN), a general carcinogen (Sharan, 1996), was also used in the study in a chronic oral administration protocol (AEBN group) over a period of 4 weeks. AEBN was prepared as described earlier (Sharan and Wary, 1992; Sharan, 1996; Pariat, 1997). Briefly, fresh betel nuts were shelled and coarsely ground. The ground betel nut was suspended in double-distilled H_2O at a concentration of 100 mg per 250 ml for 24 h at room temperature. The suspension was then filtered through Whatman no. 1 filter paper. The filtrate was lyophilized in Lyolab 3000 freeze dryer. The powder has been referred to as AEBN. It was dissolved in water at a concentration of 0.5 mg ml^{-1} and administered to mice in chronic oral administration protocol. Mice were sacrificed for analysis of different tissues primarily by cervical dislocation except when blood has to be drawn in which case it was done by chloroform anesthesia. They were sacrificed 1, 2, 3 and 4 weeks after initiation of the treatment. Age matched mice served as controls.

2.5. 3-AMINOBENZAMIDE TREATMENT

3-Aminobenzamide (3-AB) was prepared in double distilled water and was used at a dose rate of 5.45 mg kg^{-1} body weight, which is equivalent to 2 mM of 3-AB (Devi, 2001). Mice were injected with the 3-AB solution every week intraperitonially in an acute weekly injection protocol. One group of positive control mice received 3-AB alone (3-AB group). Another group of mice, maintained chronically on DMN, also received weekly 3-AB injections (DMN + 3-AB group).

2.6. PREPARATION OF GENOMIC DNA

Genomic DNA was prepared from mice spleen using a standard method for mammalian tissue (Asubel *et al.*, 1995) with some modifications.

2.6.1. Requirements:

1. Digestion buffer: 100 mM NaCl, 10 mM tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5 % (w/v) SDS, 0.1 mg/ml proteinase K. The proteinase K being labile was added fresh for every use.
2. PBS, pH 7.4, ice-cold
3. Extraction solution: Phenol: chloroform: isoamyl alcohol (25:24:1)
4. 7.5 M ammonium acetate
5. 100 % and 70 % ethanol
6. TE buffer, pH 8: 10 mM tris-Cl and 1 mM EDTA, pH8.0

2.6.2. Methodology:

Control and 1, 2, 3, or 4-week treatment groups of mice were killed by cervical dislocation or chloroform anesthesia. Spleens were taken out, weighed and frozen at – 80 °C. Nine hundred mg of the tissue was ground to powder with pre-chilled mortar and pestle. To this digestion buffer was added at a volume of 1.2 ml for 100 mg of tissue. The sample was transferred to tightly capped tubes and incubated for 12 to 18 h at 50 °C with frequent shaking. The samples were extracted with extraction solution for few minutes. This was then centrifuged for 10 min at 1700 x *g*. The top (aqueous) layer was transferred to a new tube and the volume was measured. To this, half the volume of 7.5 M ammonium acetate and two volume of 100 % ethanol were added. It was then centrifuged for 2 min at 1700 x *g*. The pellet, DNA was collect in Eppendorf tubes and washed once with 70 % ethanol. The pellet was air dried in oven at 37 °C for 15 min. The DNA was then resuspended in TE buffer at approx. 1 mg ml^{-1}.

2.7. DNase I DIGESTION OF GENOMIC DNA

Spleen cell genomic DNA from control and treated group mice were digested with DNase I enzyme to find out the structural alterations in the genomic DNA following the method standardized in the laboratory (Margison and O'Conner, 1979; Pariat, 1997; Devi, 2001).

2.7.1. Requirements:

1. DNase I stock buffer: 20 mM tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 100 µg ml^{-1} BSA and 50 % Glycerol was used to dissolve DNase I at a concentration of 1 mg ml^{-1} (equivalent to 2140 U). The solution was aliquoted and stored at –20 °C.
2. Digestion buffer: 85 mM KCl, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.5 and 5 % sucrose.
3. Stop buffer: 50 mM tris-HCl, pH 7.5, 150 mM NaCl, 15 mM EDTA and 0.3 % SDS.

2.7.2. Methodology:

Autoclaved pipette tips and Eppendorf tubes were used throughout the experiment. Five µl (28 µg) of DNA sample was taken in an Eppendorf tube and 8 µl of digestion buffer was added. To this, 3.5 µl (7.5 U) of DNase I was added. It was spun mildly and incubated for 3 min at 37 °C. Immediately after incubation, 3.5 µl of stop buffer

was added to stop the reaction. Two µl of 10 x loading buffer was added to it in preparation for agarose gel electrophoresis.

2.8. AGAROSE GEL ELECTROPHORESIS

2.8.1. *Requirements:*

1. Agarose
2. Electrophoresis buffer (TAE buffer): 40 mM tris-acetate, pH 8.42, 1 mM EDTA.
3. Loading buffer: 40 % Sucrose and 0.25 % Bromophenol blue.

2.8.2. Methodology:

Agarose gel (0.6 %) was prepared in TAE buffer and allowed to polymerize for 15-20 min at room temperature. The samples (3µl, equivalent to 3.8 µg of DNA) were loaded in the TAE buffer-submerged wells. Electrophoresis was carried out at 100 V (constant) for 60 min in a mini gel electrophoresis system (Mupid, Japan). The gel was stained in 0.7 µg ml^{-1} of Ethidium bromide for 15 min, destained in water and photographed on an UV transilluminator using DC 120 digital zoom camera attached to a PC.

2.9. PULSE-FIELD GEL ELECTROPHORESIS

2.9.1. Requirements:

1. Agarose
2. TBE buffer, 0.5 x, pH 8.3:
   (A) Stock solution: One l of 5 x TBE, pH 8.3 was prepared using Tris base (54 g), Boric acid (27.5 g) and EDTA (3.72 g).
   (B) Working solution: 0.5 x TBE (0.05 M), pH 8.3 was prepared from the stock by dilution.
3. Loading buffer: 40 % Sucrose and 0.25 % Bromophenol blue.

2.9.2. Methodology:

Agarose gel (1.2 %) was prepared by dissolving 1.3 g of agarose in 110 ml of 0.5 x TBE, pH 8.3. The gel was allowed to polymerize in the gel casting kit on the horizontal gel platform. Samples (3 µl, equivalent to 3.8 µg of DNA) were loaded in wells. Electrophoresis was carried out for 4 h at 200 volts (constant) with pulse time of 25 sec at 8 °C. After electrophoresis the gel was stained in 0.7 µg ml^{-1} Ethidium bromide for 20-25 min, destained in water and then photographed on a UV mini transilluminator using DC 120 digital zoom camera attached to a PC.

2.10. ISOLATION OF ADP-RIBOSE POLYMERS

ADP-ribose polymer, for use as antigen to raise polyclonal antibodies against it, was isolated following the method standardized in the laboratory (Saikia, 1996; Devi, 2001). Briefly, mice spleens were removed and washed in PBS, pH 7.4. The spleen cells were homogenized in 4 ml PBS, centrifuged at 1,300 x *g* for 15 min. The pellet was dispersed in 10 % TCA by vortexing. The suspension was left in freezer for 30 min and centrifuged at 20,000 x *g* for 15 min. The supernatant was saved while 2 ml

of tris-glycine buffer (0.1 M, pH 10.5) was added to the pellet, vortexed, incubated at 37 °C for 1 h with occasional shaking and recentrifuged at 20,000 x *g*. The 20,000 x *g* supernatants were pooled which contained the heterogeneous ADP-ribose polymers. A part of it was used for the estimation of DNA, RNA and protein to verify success of isolation. The isolate was desalted by passing it through sephadex G-25 column using 0.03 M tris barbiturate buffer, pH 7.2 containing 6 M urea and 0.1 % SDS at a flow rate of approx. 1 ml 5 min^{-1}. The fractions showing peak absorbance at 490 nm were pooled. A part of it was again subjected to DNA, RNA and protein estimations. The eluent was dialyzed extensively against double distilled water. The dialyzed solution was fan dried to concentrate the polymer and lyophilized. Estimations of DNA, RNA and protein were performed again. This fraction was stored refrigerated until its use as antigen to immunize the rabbit.

2.11. PREPARATION OF POLYCLONAL ANTIBODIES AGAINST ADP-RIBOSE POLYMER

The method of immunization, standardized in the laboratory (Rosenberg, 1996; Humtsoe, 2000; Devi, 2001), was essentially followed. Briefly, an emulsion of antigen: Freund's complete adjuvant (1:1) was prepared and a healthy, young rabbit was given primary immunization shots sub-cutaneously on different spots on the back of rabbit with approx. 0.5 ml of emulsion per spot. After 4 weeks, secondary immunization was done. For first booster immunization, an emulsion of 0.5 ml of antigen (1 mg ml^{-1}) and 0.5 ml of Freund's incomplete adjuvant (1:1) was used. The second booster dose was given after 2 weeks of the first booster. After 10 days of the second booster dose the rabbit was bled from the marginal veins of the ears.

Blood was collected in a measuring tube pre-rinsed with 0.9 % NaCl. The blood was allowed to stand for 2-3 h at room temperature to clot. The serum was collected in centrifuge tube and spun at 5000 × *g* for 10 min to remove any remaining red cells and debris. The serum was stored refrigerated in fresh tubes. The process of bleeding was continued after every 2 weeks of the last booster injection. The serum immunoglobulins were then precipitated using ammonium sulphate. It was solidified, lyophilized and stored at 0 °C.

2.12. SPECIFICITY OF THE RAISED POLYCLONAL ANTIBODIES

The specificity of the polyclonal antibody (Ab) against ADP-ribose polymer antigen (Ag) was tested by Ouchterlony immunodiffusion (Ouchterlony, 1949). Briefly, agarose (1 % w/v) dissolved in 0.9 % NaCl solution containing 0.02 % sodium azide, was poured on clean glass slides and allowed to polymerize. Three wells were punched on the gel approximately 1 cm apart. Ab was loaded in the middle well. In the other 2 wells, Ag (ADP-ribose polymer) and BSA were loaded, respectively. Several such slides were prepared with serially diluted antigen and antibody preparations. The slides were kept under humid conditions at 37 °C for more than 24 h or until precipitin line was visible. The slides were stained with coomassie brilliant blue for few hours and then destained before taking photograph using a Kodak digital camera.

2.13. SNAKE VENOM PHOSPHODIESTERASE DIGESTION OF ADP-RIBOSE

Snake venom phosphodiesterase (SVP) is known to cleave the pyrophosphate bonds endonucleolytically. Thus, it specifically degrades the ADP-ribose polymers (Sugimura, 1974). In order to test the specificity of the raised polyclonal antibody against ADP-ribose polymer, SVP was first used to degrade the PAR from Ag, poly ADP-ribose. The SPV digested Ag was subjected to immunodiffusion assay as described below (§ 2.21.).

2.13.1. Methodology:

Blood lymphocyte histones (§ 2.17.) from normal mice were taken in different Eppendorf tubes at a concentration of 4 μg 100 μl^{-1}. One hundred μl of freshly prepared SVP (2 mg ml^{-1}) was added to each tube. The tubes were incubated in a water bath at 37 °C for 15, 20, 25, 30, 35 and 40 min for SPV to progressively degrade the ADP-ribose polymers. An SPV-untreated whole homogenate sample under similar conditions was used as control. One hundred μl of the sample was then slot blotted onto nitrocellulose membrane (NC; 0.45 μ) under vacuum and subjected to immunoprobing (§ 2.21.). The NC membrane was then dried, scanned, quantified and analyzed using imaging densitometer GS-690 with molecular analyst PC software 1.5.

2.14. ISOLATION OF BLOOD LYMPHOCYTES

Lymphocytes were isolated from freshly drawn blood of normal (control) and treated mice using Biocoll-based gradient centrifugation using heparin treated syringes (Boyum, 1968; Keller *et al.*, 1982). Equal volumes of heparinized blood and RPMI-1640 were mixed. Biocoll solution was poured in centrifuged tubes and an equal volume of blood-RPMI-1640 mixture was added to it gently through the wall of centrifuged tube by keeping the tube in a slanting position. The tube was then centrifuged at 400 x *g* for 40 min. The lymphocyte layer was collected using syringe. Lymphocytes were washed with PBS, spun at 250 x *g* for 10 min and resuspended in the left over PBS, pH 7.4. Cell number was counted.

2.15. COUNTING OF CELL NUMBER

The number of cells per ml of cell suspension was determined using Bürker cell counting chamber under phase contrast microscope. The cell suspension was diluted 50 x and loaded onto the Bürker cell. Number of cells in 25 small chambers was counted under microscope. The following formula was used to calculate the total number of cells per ml of suspension:

Number of cells per ml = Number of cells in 1 small chamber x 25,000 x 50

2.16. PREPARATION OF HOMOGENATE FOR ASSAY OF POLY-ADP RIBOSYLATION

Whole homogenates (WH) of liver, spleen cells, BMC and blood lymphocytes were prepared for different experiments. For the preparations of WH either all cells from 6

spleens in 4 ml PBS, all BMC from 12 femurs in 2 ml PBS, 0.15 g finely sliced liver in 3 ml PBS or 6 X 10^6 blood lymphocytes were used.

2.16.1. Requirements:

1. Lysis buffer: It contained 20 mM tris buffer, pH 8.0, 10 mM NaCl, 0.5 % triton X-100, 5 mM EDTA and 3 mM $MgCl_2$.
2. Phosphate buffered saline (PBS), pH 7.4.

2.16.2. Methodology:

Whole homogenates (WH) of tissues were prepared by the method of Rosenberg (1996) with some modifications. Tissues were excised out of control or treated mice killed by cervical dislocation or chloroform anesthesia. The excised tissues were washed with PBS and cell suspensions made in PBS. Cell suspension was then transferred to a centrifuged tube and pelleted by centrifugation at 200 x *g* for 10 min at 4 °C. Cells in the pellet were gently dispersed or piece of liver was finely sliced before adding the ice-cold lysis buffer (1.5 ml) and vortexed. It was left on ice for 30 min. It was then spun at 5,000 x *g* for 10 min at 4 °C. The supernatant was used for various experiments after estimation of proteins by Bradford's method (§ 2.24.).

2.17. ISOLATION OF HISTONES

Histone proteins were isolated from spleen cells, liver and blood lymphocytes. Thirty million (30 x 10^6) spleen cells, 12 x 10^6 lymphocyte cells or 0.3 g of liver in 4 ml PBS was used for the isolation. The method is based on West and Bonner (1980) as adapted in our laboratory (Saikia *et al.*, 1998).

2.17.1. Requirements:

1. SPC buffer: 1 mM potassium phosphate buffer, pH 6.8 containing 0.32 M sucrose and 1 mM $CaCl_2$.
2. 1 M DTT
3. Bench HCl
4. β-mercaptoethanol (β-ME)
5. Urea
6. 0.2 % phenolphthalein
7. Bench NH_3
8. Glacial acetic acid (GAA)
9. PBS, pH 7.4

2.17.2. Methodology:

Control or treated mice were sacrificed by cervical dislocation or chloroform anesthesia. Liver and spleen were excised out. Simultaneously blood was drawn from mouse heart and blood lymphocytes prepared (§ 2.14.). Spleen cell and blood lymphocyte cell suspensions were made in PBS and were counted (§ 2.15.). The WH was put in eppendorf tubes. It was spun for 15 sec in microcentrifuge and the supernatant discarded. To this 50 µl PBS, 500 µl SPC, 20 µl concentrated HCl and 12 µl β-ME were added and vigorously vortexed. The mixture was left in ice for 1 h and then centrifuged for 5 min at 10,000 x *g*. Supernatant was collected and its volume

measured. Urea was added at a concentration of 0.75 g ml^{-1} supernatant and dissolved by vortexing. Now 5 µl of phenolphthalein was added. To this, 0.05 x volume of 1 M DTT and 50 µl NH_3 were added. Characteristic pink color appeared indicating completion of the isolation of histones. GAA was now added to a final concentration of 1 M. The protein content of the isolate was estimated using Bradford's method (§ 2.24.).

2.18. SLOT-BLOTTING

2.18.1. Requirements:

1. Tris buffered saline (TBS): 20 mM Tris, pH 7.5, 500 mM NaCl.
2. PVDF membrane
3. BioRad Bio-Dot® SF microfiltration apparatus
4. Filter papers

2.18.2. Methodology:

All samples for slot blotting were incubated in boiling water bath for 5 min to inactivate endogenous alkaline phosphatase in order to avoid its interference in a later step. The samples were diluted with H_2O to a final concentration of 3 µg proteins in 100 µl. One hundred µl samples were loaded in each well and slot blotted on activated PVDF (pre-wetted in TBS, pH 7.5) membrane by applying gentle vacuum through Bio-Dot®SF microfiltration apparatus. Usually it took 30-45 min to complete slot blotting. The membrane was then either subjected to immunoprobing for PAR (§ 2.21.) or India ink staining for total protein (§ 2.22.).

2.19. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Samples for analysis of protein profile or Western blotting were first subjected to SDS-PAGE. The method is essentially based on Laemmli (1970) as adapted in our laboratory.

2.19.1. Requirements:

1. Separating gel: For preparation of separating gel, various working solutions were mixed in ratios shown in the table below, degassed and poured.

Gel component	*Volume (ml) for 12 % gel*
Monomer (30 % w/v)	4.0
1 M tris-HCl, pH 8.8	3.7
H_2O	2.21
10 % SDS	0.05
TEMED	0.01
APS	0.03

2. Stacking gel: For preparation of separating gel, various working solutions were mixed in ratios shown in the table below, degassed and poured.

Gel component	*3 % gel (ml)*
Monomer (30 % w/v)	0.5
1 M tris-HCl, pH 6.8	0.625
H_2O	3.8
10 % SDS	0.05
TEMED	0.012
APS	0.013

3. Sample buffer (5 x): It consisted of the following:

Components	*Quantity*
Tris-HCl buffer, pH 6.8	5 ml
SDS	0.5 g
Sucrose	5 g
β-ME	0.25 ml
Bromophenol blue	0.005 g

The final volume was made up to 10 ml with double-distilled H_2O and was stored at room temperature.

4. Electrophoresis buffer, pH 8.3 (10 x): The electrophoresis buffer was made up of:

Components	*Quantity*
Tris	30 g
Glycine	144 g
SDS	10 g

The constituents were dissolved in 850 ml H_2O and the pH was adjusted to 8.3. The final volume was made up to 1 l with H_2O.

5. Coomassie brilliant blue (CBB) stain (0.25 % w/v): CBB (2.50 g) was dissolved in a mixture of 100 ml concentrated acetic acid and 400 ml methanol. The final volume was made up to 1 l with H_2O. It was then filtered through Whatman #1 filter paper in a dark reagent bottle and stored at room temperature.

6. Destaining solution: Concentrated acetic acid (100 ml) was slowly added to 400 ml of methanol and the final volume was made to 1 l with H_2O.

2.19.2. Methodology:

Part of the separating gel 12 % components (without TEMED and APS) were mixed in a vacuum flask and degassed for 10 min using rotary vacuum pump. Meanwhile, the BioRad Mini-PROTEAN® II electrophoresis cell gel-casting set-up was assembled. TEMED and freshly prepared APS were added and mixed properly with the degassed components and the mixture was poured in between the gel casting glass plates to a level of approx. 1 cm below the tooth of the comb to be used to form the wells. It was immediately overlaid with H_2O. The gel usually polymerized in 30-40

min at room temperature. After polymerization, the overlaying water was discarded and the surface of separating gel was dried. The components for stacking gel was prepared in the same way as was done for separating gel using the composition as shown in the table above. Before the stacking gel was cast, the comb was placed between the glass plates such that it sits on one of the spacer of the gel casting assembly and tilting on other side. From the tilted end stacking gel mixture was poured gently avoiding trapping of any air bubble and then the comb was allowed to firmly sit on the spacer. The stacking gel usually polymerized in about 50-60 min. Comb was removed from the cast gel and the well rinsed with H_2O to remove traces of un-polymerised gel components.

Samples for SDS-PAGE were prepared by mixing sample and sample buffer in a ration of 4:1 and incubated in a boiling water bath for 5 min. During this period, PAGE apparatus was assembled for electrophoresis. Fifteen to 20 µl samples were loaded in each well. The gel was run using 1 x running buffer at a constant volt of 200 or 150 V for 45 or 60 min, respectively. The gel was removed and either stained in coomassie brilliant blue (CBB) or subjected to Western blotting (§ 2.20.).

The CBB staining was done overnight. The gel was destained completely and then photographed using Kodak digital camera. The gels were then dried in BioRad gel dryer attached to a vacuum pump and a vapor trap.

2.20. WESTERN BLOTTING

The proteins resolved on SDS-PAGE gels were Western blotted onto PVDF membrane for immunoprobing of poly-ADP-ribosylated proteins.

2.20.1. Requirements:

1. Towbin buffer: 25 mM tris-Cl, pH 8.3, 192 mM glycine and 20 % methanol.
2. PVDF membrane
3. BioRad Mini trans-blot® electrophoretic transfer apparatus

2.20.2. Methodology:

After the protein samples were subjected to SDS-PAGE, the gels were taken out and incubated in Towbin buffer for 10-15 min for equilibration. While the SDS-PAGE was going on, the PVDF membranes were activated. It was rinsed with 100 % methanol for 10 sec. It was then wetted by incubating it in double distilled water. This was important for effective binding of proteins to the membrane. Finally it was incubated in Towbin buffer for 5-10 min. Meanwhile, the Western blot apparatus was assembled for the transfer of the proteins to the PVDF membrane by Western blotting technique. The transfer was carried out in chilled Towbin buffer using BioRad Mini trans-blot® electrophoretic transfer cell at 100 V (constant) for 60 min. Bio-Ice cooling unit was also used to maintain appropriate buffer temperature. The electroblotted membranes were then processed for immunodetection (§ 2.21.) or India ink staining (§ 2.22.).

2.21. IMMUNOPROBING

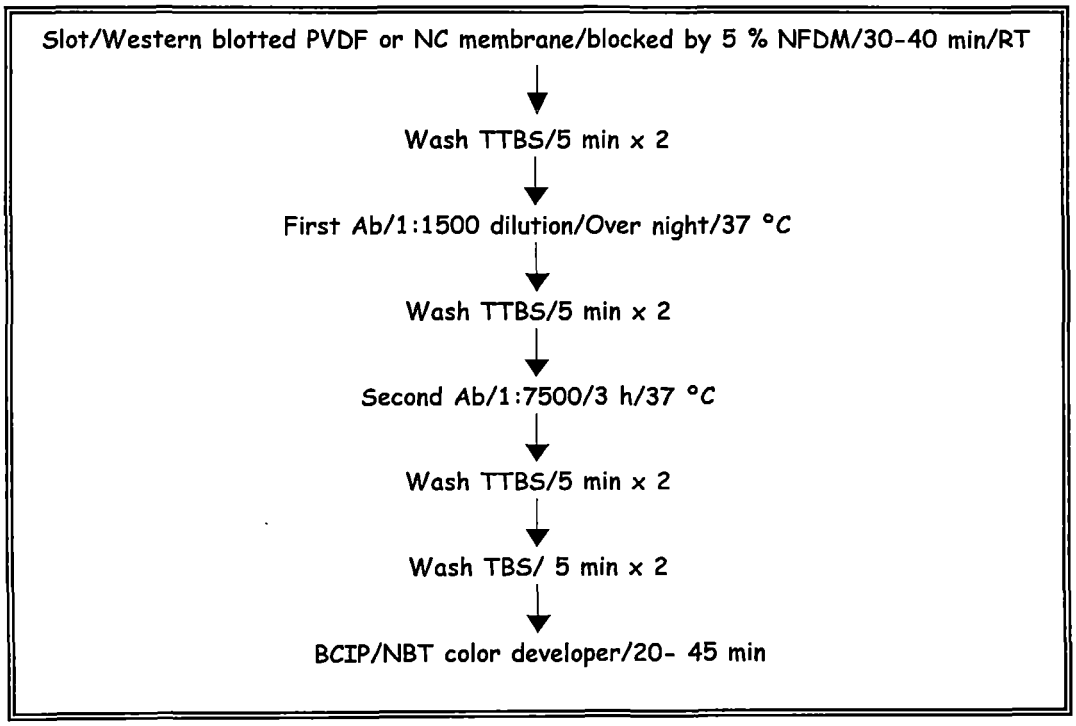
The slot or Western blotted PVDF or NC membranes were processed for ELISA-based immunoprobing for assay of PAR of proteins. Standard protocol for immunodetection has been used with some modifications (Nozaki *et al.*, 1994; Sharan *et al.*, 1998b).

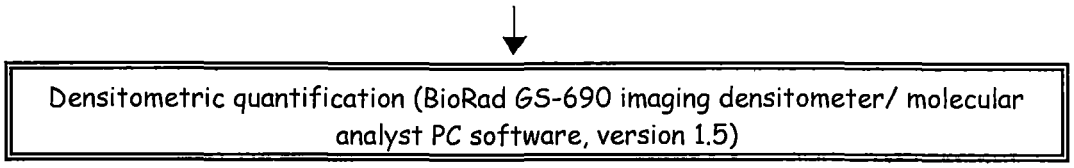
2.21.1. Requirements:

1. Tris buffered saline (TBS), pH 7.5: It consisted of 20 mM Tris-Cl buffer (pH 7.5) with 500 mM NaCl.
2. Tween-20, tris buffered saline (TTBS), pH 7.5: This comprised TBS with 0.05 % Tween-20.
3. Blocking solution: This was made by dissolving 5 % non-fat skimmed dry milk (NFDM) in TBS.
4. First antibody (polyclonal anti ADP-ribose) solution: Anti-ADP-ribose polyclonal antibody raised in rabbit and stored as lyophilized powder (§ 2.11.) was dissolved in blocking solution at a concentration of 2 mg ml^{-1}. The effective dilution was 1:1500.
5. Second antibody solution: Anti-rabbit IgG-alkaline phosphatase conjugate at a dilution of 1:7500 was used for this purpose.
6. BCIP/ NBT color developer: This is the substrate of the enzyme alkaline phosphatase. This is acted upon by the alkaline phosphatase of the second antibody conjugate and gives a characteristic yellowish-violet colour to the poly-ADP ribosylated protein bands on the membranes.

2.21.2. Methodology:

The immunoprobing method standardized in the laboratory was used (Sharan *et al.*, 1998b). Essentially it consisted of the following steps as shown in the flow chart below.





2.22. INDIA INK STAINING

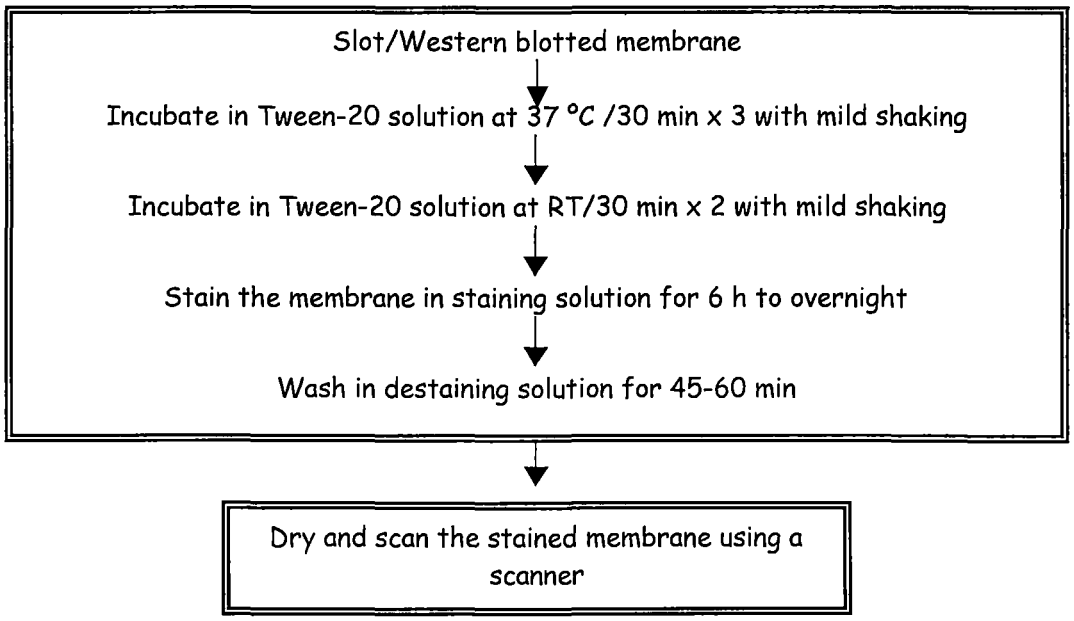
The slot and Western blotted membranes were also stained with India ink to monitor the total protein present in slots or Western blots.

2.22.1. Requirements:

1. Staining solution: It contained 0.2 % Rotring India ink with 0.3 % Tween-20 in 25 ml PBS, pH 7.4. To get this 50 µl of ink and 75 µl of Tween-20 were added in 25 ml of PBS.
2. Destaining solution: This consisted of 0.3 % (v/v) Tween-20 in PBS, pH 7.4. To prepare 1 l destaining solution, 3 ml of Tween-20 was added in 1 l of PBS.

2.22.2. Methodology:

The following steps completed the process of ink staining.



2.23. ESTIMATION OF DNA

Amount of DNA in the experimental samples was estimated using diphenylamine (DPA) test (Burton, 1968) with some modifications. Commercially available salmon testis DNA at a concentration of 2 mg ml^{-1} was used as a standard.

2.23.1. Requirements:

DPA reagent was prepared by dissolving 1.5 g of DPA in 100 ml of glacial acetic acid and 1.5 ml of concentrated H_2SO_4. This was stored at room temperature.

2.23.2. Methodology:

Ten to 100 µl of the standard DNA solution was taken in different test tubes. The volume was made up to 100 µl by adding H_2O. Two ml of DPA reagent was added in each tube and vortexed. Test samples were prepared similarly. Blank was made with 100 µl water and 2 ml of DPA reagent. All the test tubes were incubated in boiling water bath for 10 min. After cooling it down, absorbance was read at 595 nm against the blank. A standard curve was prepared and the concentration of the test sample was calculated.

2.24. ESTIMATION OF PROTEINS

The estimation of proteins in the experimental samples was done by Bradford's method (1976) with some modifications. BSA solution at a concentration of 1 mg ml^{-1} was used as a standard.

2.24.1. Requirements:

A stock solution of Bradford reagent was prepared by dissolving 100 mg of Coomassie brilliant blue G-250 in 50 ml of 95 % ethanol. To this 100 ml of 85 % (w/v) phosphoric acid was added and mixed properly. This was kept in a dark bottle and stored refrigerated. One hundred ml of working solution was prepared by mixing 15 ml of the stock solution and 85 ml of H_2O. The working solution was filtered through Whatman no. 1 filter paper and the filtrate was stored refrigerated. For each use the working solution was freshly prepared.

2.24.2. Methodology:

Aliquots of 10-100 µl of BSA solution were taken in different test tubes. The volume was made up to 100 µl by adding double distilled water. Five ml of working solution was added in each tube. It was gently mixed and incubated in room temperature for 5 min. Similarly the test samples were also prepared. Blank was prepared by adding 100 µl of water and 5 ml of the working solution. It was also incubated for 5 min. Absorbance of the samples was read at 595 nm against the blank solution. A standard curve was made and the concentration of the test samples was calculated from this.

2.25. ESTIMATION OF RNA

Estimation of RNA in the experimental samples was done by Orcinol method (Merchant *et al.*, 1969) with some modifications. Commercially available RNA (type III from Bakers yeast) was used as a standard at a concentration of 1 mg ml^{-1}.

2.25.1. Requirements:

Orcinol reagent was prepared by dissolving 1 g of orcinol in 100 ml of concentrated HCl containing 0.5 g of ferric chloride. The reagent was stored refrigerated in a dark bottle. This was used up to 4 weeks.

2.25.2. Methodology:

Aliquots of 10-100 µl of standard RNA solution were taken in different test tubes. The volume was made up to 100 µl by adding water. Two ml of Orcinol reagent was added in each test tubes and vortexed. Test samples were prepared similarly. Blank was prepared by adding 100 µl of water into 2 ml of Orcinol reagent. The test tubes were incubated in boiling water bath for 20 min. Absorbance was read at 660 nm against the blank after cooling the tubes. A standard curve was prepared and the concentration of the test samples was calculated from the graph.

2.26. QUANTITATIVE AND STATISTICAL ANALYSIS

A gel documentation and analysis system, Kodak digital science 1 D image analysis software using DC120 zoom digital camera was employed to analyze the electrophoresis gels. The immunoprobed slot- and Western-blots were quantified using imaging densitometer GS-690 with molecular analyst PC software 1.5. Pixel density of the bands (% of control) was used to analyze the PAR level of the total cellular proteins and histones in different experimental conditions. Statistical calculations, Student's t-test, *etc.* were done using Microsoft Excel program. Origin software (version 3.5) was used to plot the graph. Graph was plotted using PAR (% of control)/mean ± SEM *versus* period of treatment for all the data. Data showing $P \leq 0.01$ and $P \leq 0.05$ were taken as statistically significant.

Chapter 3

RESULTS

3.1. EFFECTS ON GENOMIC DNA

3.1.1. DMN treatment

Genomic DNA was isolated from spleen cells of normal and DMN exposed mice (§ 2.6.). The genomic DNA was subjected to DNase I digestion (§ 2.7.) and loaded on a 0.6 % agarose gel for electrophoresis (§ 2.8.). Fig. 5 below shows the result. The genomic DNA was progressively more digested by DNase I as DMN exposure period increased.

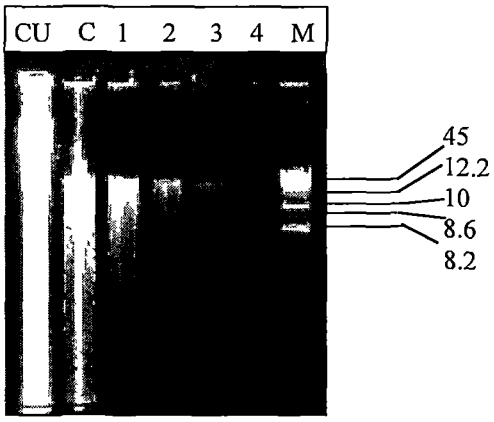


Figure 5. Agarose gel electrophoresis of genomic DNA isolated from spleen cells of mice exposed to DMN for up to 4 weeks and digested by DNase I; lanes: CU - control undigested, C - control, 1, 2, 3 and 4 (DMN treatment period in week, respectively) and M - molecular weight marker in kDa.

3.1.2. DMN + 3-AB treatment

Genomic DNA was isolated from spleen cells of normal and DMN + 3-AB exposed mice (§ 2.6.). The genomic DNA was subjected to DNase I digestion (§ 2.7.) and loaded on a 0.6 % agarose gel for electrophoresis (§ 2.8.). Fig. 6 below shows that the genomic DNA was very extensively and progressively more digested by DNase I as DMN + 3-AB exposure period increased.

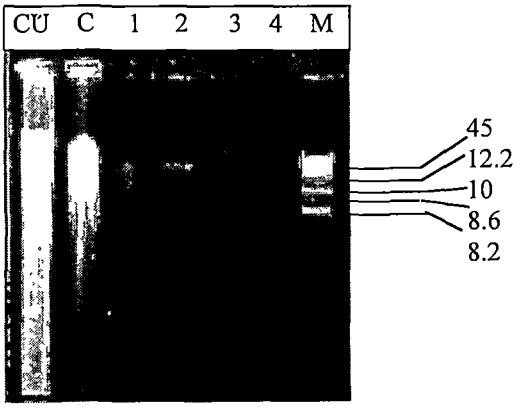


Figure 6. Agarose gel electrophoresis of genomic DNA isolated from spleen cells of mice exposed to DMN + 3-AB for up to 4 weeks and digested by DNase I; lanes: CU - control undigested, C - control, 1, 2, 3 and 4 (DMN + 3-AB treatment period in week, respectively) and M - molecular weight marker in kDa.

3.1.3. AEBN treatment

Genomic DNA isolated was from spleen cells of normal and AEBN exposed mice (§ 2.6.), subjected to DNase I digestion (§ 2.7.) and loaded on a 0.6 % agarose gel for electrophoresis (§ 2.8.). The digestion of AEBN treated genomic DNA by DNase I was also progressive as AEBN exposure period increased, particularly in the 3rd and 4th weeks of treatment (Fig. 7).

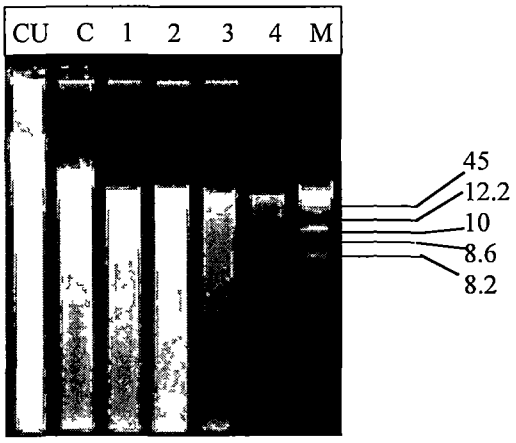


Figure 7. Agarose gel electrophorogram of genomic DNA from isolated spleen cells of mice exposed to AEBN for up to 4 weeks and digested by DNase I; lanes: CU - control undigested, C - control, 1, 2, 3 and 4 (AEBN treatment period in week, respectively) and M - molecular weight marker in kDa.

3.2. PULSE FIELD GEL ELECTROPHORESIS (PFGE) OF DMN TREATED GENOMIC DNA AFTER DNase I DIGESTION

Genomic DNA was isolated from spleen cells of normal and DMN exposed mice (§ 2.6.), digested by DNase I (§ 2.7.) and loaded on a 1.2 % agarose gel for pulse field gel electrophoresis (§ 2.9.). Fig. 8 below shows the result. Similar pattern of progressive increasing digestion of the genomic DNA was obtained with increase in DMN exposure period.

Figure 8. Pulse field gel electrophorogram of genomic DNA isolated from spleen cells of mice exposed to DMN for up to 4 weeks and digested by DNase I; lanes: CU - control undigested, C - control, 1, 2, 3 and 4 (DMN treatment period in week, respectively) and M - yeast artificial chromosome marker.

3.3. IMMUNOGENICITY OF THE ANTISERUM

The immunogenicity of the raised antiserum against the antigen (Ag), poly-ADP-ribose, was confirmed by Ouchterlony immunodiffusion assay (§ 2.12.). Fig. 9 shows the characteristic precipitin line between the well with the Ag, poly-ADP-ribose, and the antiserum against it. No such line was observed between antiserum and BSA.

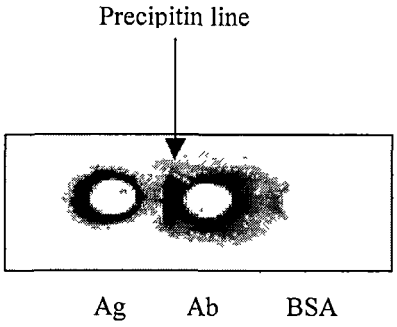


Figure 9. Ouchterlony immunodiffusion: The central well contain polyclonal anti poly ADP-ribose antibody (Ab), left well had ADP-ribose polymer (Ag) and the right well, BSA. The slide was incubated under moist condition at 37°C for 36-48 h. The precipitin line can only be seen between the well with Ag and Ab specific to each other.

3.4. SPECIFICITY OF THE ANTISERUM

The specificity of the raised antibody was determined by observing its interaction with Ag after digesting the ADP-ribose polymers of blood lymphocyte histones (§ 2.17.) from normal mice by snake venom phosphodiesterase I (SVP) (§ 2.13.). The sample was slot blotted on PVDF membrane (§ 2.18) and immunoprobed (§ 2.21.). From the result in Fig. 10 it is clear that with increasing time of SVP digestion of poly ADP-ribose on histone proteins, the colour of slots progressively became weaker (row 1 to 6) following immunoprobing. No PAR was detectable from row 4 onwards.

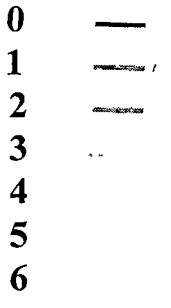


Figure 10. Specific immunological interaction between Ab (against poly ADP-ribose) and the Ag (ADP-ribose polymers) as shown by blood lymphocyte histones after slot blotting and immunoprobing. Row 0: SVP undigested (control), 1 to 6 – SVP digested Ag (15, 20, 25, 30, 35 and 40 min, respectively at 37 °C).

3.5. INFLUENCE OF DMN ON TOTAL PAR OF CELLULAR PROTEIN

Whole homogenate (WH) was prepared from liver, spleen cells (SC), bone marrow cells (BMC) or blood lymphocytes of normal and DMN treated mice (§ 2.16.), and

slot blotted on PVDF membrane (§ 2.18.). Lane I shows India ink stained membrane (§ 2.22.) while lane II shows immunoprobed replica of it (§ 2.21.) (Fig. 11).

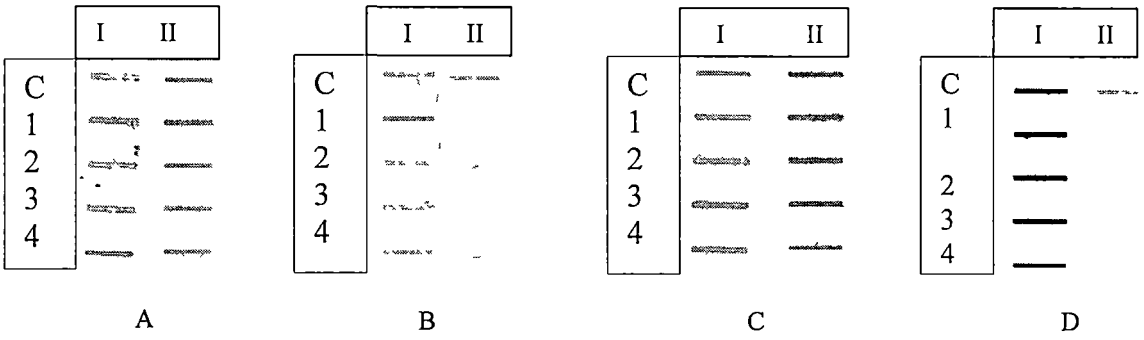


Figure 11. Whole homogenates of different tissues after they were subjected to slot blotting on PVDF membrane and stained by India ink for total protein quantification (I) or its replica immunoprobed for PAR (II) Panels A - liver, B - spleen cells, C - bone marrow cells and D - blood lymphocytes; rows C - control, 1, 2, 3 and 4 (DMN treatment period in week).

Fig. 11 above shows the result. There was a significant reduction in the PAR signal in the treated samples when compared with the control (lane II), especially in 3rd and 4th weeks of treatment. Lane I shows that approximately equal amount of proteins have been slotted.

3.6. INFLUENCE OF DMN + 3-AB ON TOTAL PAR OF CELLULAR PROTEINS

Whole homogenates (WH) were prepared from liver, SC, BMC or blood lymphocytes of normal and DMN + 3-AB treated mice (§ 2.16.). The protein samples were slot blotted on PVDF membrane (§ 2.18.). Lane I shows India ink stained membrane (§ 2.22.) while lane II shows immunoprobed replica of it (§ 2.21.).

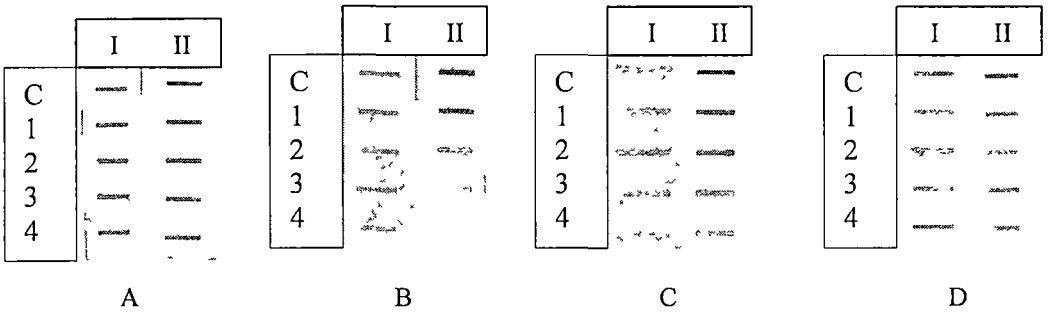


Figure 12. Whole homogenate of different tissues after they were subjected to slot blotting on PVDF membrane and stained by India ink for total protein quantification (I) or its replica immunoprobed for PAR (II). Panels A - liver, B - spleen cells, C - bone marrow cells and D - blood lymphocytes; rows C - control, 1, 2, 3 and 4 (DMN + 3-AB treatment period in week).

Fig. 12 shows that there was relatively greater reduction in the PAR signal of the treated samples when compared with their respective controls (lane II), especially in 2nd, 3rd and 4th weeks of DMN + 3-AB treatment. Amount of proteins slotted was approx. equal in each case (lane I).

3.7. INFLUENCE OF DMN ON TOTAL PAR OF HISTONE PROTEINS IN VARIOUS TISSUES

Histone proteins were prepared from liver, SC or blood lymphocytes of normal and DMN treated mice (§ 2.17.). The samples were slot blotted on PVDF membrane (§ 2.18.). Lane I shows India ink stained membrane (§ 2.22.) while lane II shows immunoprobed replica of it (§ 2.21.).

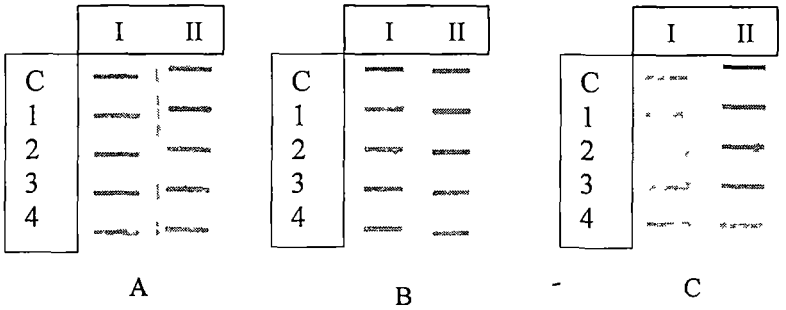


Figure 13. Histones of various tissues after they were subjected to slot blotting on PVDF membrane. The membranes were stained by India ink for total protein quantification (I) or its replica immunoprobed for PAR (II). Panels A - liver, B - spleen cells, C - blood lymphocytes; rows C - control, 1, 2, 3 and 4 (DMN treatment period in week).

Fig. 13 shows the result. There was reduction in the PAR signal of the treated samples when compared with their controls (lane II), especially in 3rd and 4th week of DMN treatment while total proteins slotted were quite identical (lane I).

3.8. INFLUENCE OF AEBN ON TOTAL PAR OF CELLULAR PROTEINS

WH were prepared from SC, BMC or blood lymphocytes of normal and AEBN treated mice (§ 2.16.). The protein samples were slot blotted on PVDF membrane (§ 2.18.). Lane I shows India ink stained membrane (§ 2.22.) while lane II shows immunoprobed replica of it (§ 2.21.) (Fig. 14).

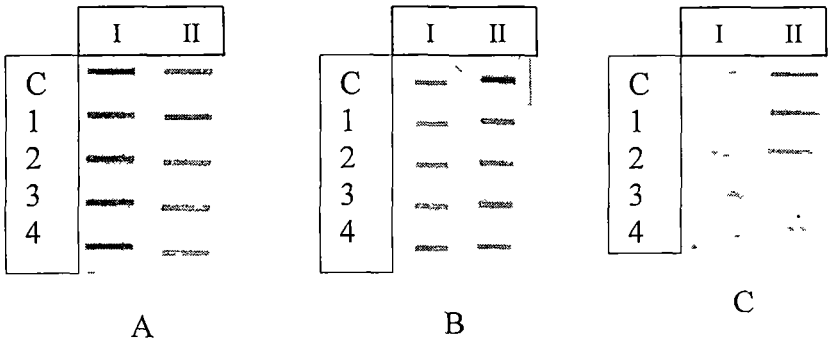


Figure 14. Whole homogenates of various tissues after the samples were subjected to slot blotting on PVDF membrane and stained by India ink for total protein quantification (I) or its replica immunoprobed for PAR (II). Panels A - spleen cells, B - bone marrow cells and C - blood lymphocytes; rows C - control, 1, 2, 3 and 4 (AEBN treatment period in week, respectively).

Fig. 14 shows the results. Progressive reduction in the PAR signal of the AEBN treated samples as compared to the control (lane II) was quite similar to the case after DMN or DMN or 3-AB exposures.

3.9. QUANTITATIVE ANALYSIS OF TOTAL PAR

3.9.1. Influence of DMN

I) Liver:

The band intensities were quantified densitometrically (§ 2.26.) to obtain 'Adj Volume OD x mm^2' which represents the mean intensity or pixel density of bands. Results of all immunodetection experiments for total PAR of liver proteins in control and DMN treated mice (§ 2.18.) were pooled. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table I). The statistical significance of difference between control and DMN exposed groups was calculated at $P \leq 0.01$ and $P \leq 0.05$. The result is shown in Table I below.

Table I: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the data are significantly different.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Exp. 6 (%)	Exp. 7 (%)	Exp. 8 (%)	Exp. 9 (%)	Mean ± SEM	Significance	
											$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100	100	100	100	100	100 ± 0.00		
1	85.01	68.31	67.56	39.41	61.73	57.97	77.12	70.72	76.02	67.10 ± 4.40	S	S
2	67.56	50.70	77.43	28.80	49.37	37.37	89.51	54.71	35.09	54.50 ± 6.76	S	S
3	55.08	39.46	58.70	28.63	32.82	36.58	66.29	34.56	38.99	43.45 ± 4.38	S	S
4	35.69	31.79	47.43	29.82	20.61	22.12	83.31	30.77	37.59	37.68 ± 6.30	S	S

The plot of the data is given in Fig. 15 below. There is a clear negative correlation between the level of total PAR in liver and the period of DMN treatment. In the 4th week of treatment the total PAR was reduced to about 60 % of the control.

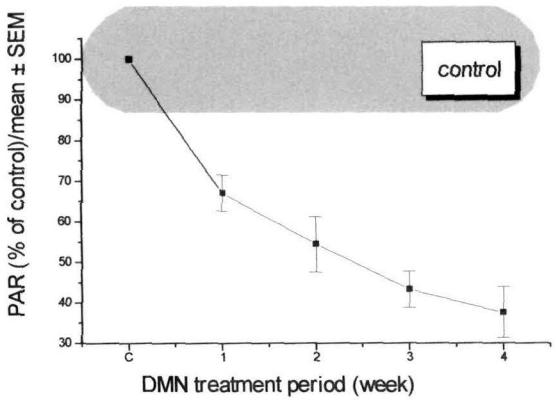


Figure 15. Relationship between total PAR of cellular proteins of liver of control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN treatment in week.

II) Spleen:

Results of immunodetection for total PAR of proteins of SC in control and DMN treated samples (§ 2.18.) were quantified densitometrically (§ 2.26) as done in case of liver. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table II).

Table II: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	69.29	99.84	64.14	78.66	77.98 ± 7.78	NS	NS
2	60.85	85.20	49.07	51.34	61.61 ± 8.26	NS	S
3	34.87	67.44	41.01	35.65	44.74 ± 7.68	S	S
4	24.43	37.82	37.83	33.26	33.33 ± 3.15	S	S

Upon plotting the data in a graph, again a straight but negative correlation was found between the level of total PAR in SC and the period of DMN treatment. In the 4th week of treatment, the reduction was about 70 % when compared with the control.

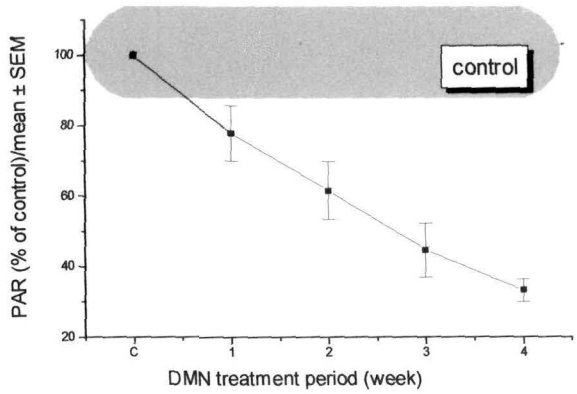


Figure 16. Relationship between total PAR of cellular proteins of spleen cells in control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN treatment in week.

III) BMC:

Using similar strategy, the total PAR of BMC proteins were quantified densitometrically (§ 2.26.) in control and DMN exposed mice.

Table III: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the data are significantly different.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Exp. 6 (%)	Mean ± SEM	Significance	
								$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100	100	100 ± 0.00		
1	69.98	88.63	93.45	71.31	60.33	69.65	75.55 ± 5.18	S	S
2	55.57	74.59	94.41	72.37	51.32	55.19	67.23 ± 6.71	S	S
3	41.77	81.35	62.87	60.07	62.76	42.25	58.51 ± 6.07	S	S
4	25.61	30.87	40.45	42.88	26.36	35.43	33.60 ± 2.94	S	S

The results are summed up in Table III. The statistical significance of the data was calculated at $P \leq 0.01$ and $P \leq 0.05$. Upon plotting the data, in case of BMC also following DMN exposure inhibition of total PAR was observed. In the 4th week of treatment was about 70 % reduction in the level of PAR when compared with the control (Fig. 17).

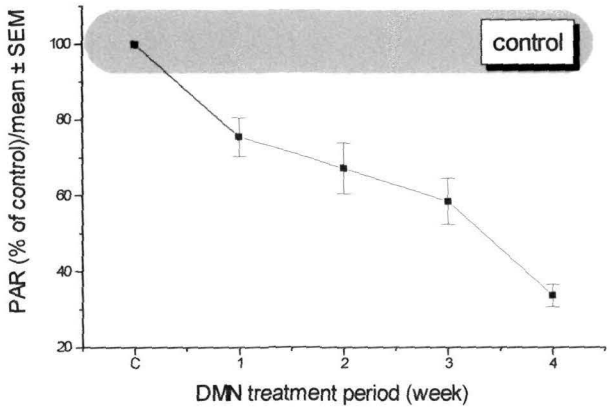


Figure 17. Relationship between total PAR of cellular proteins of BMC in control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN treatment in week.

IV) Blood lymphocytes:

The total PAR of blood lymphocyte proteins of control and DMN treated mice (§ 2.18.) were similarly quantified densitometrically (§ 2.26.). The results of individual experiments have been shown in Table IV.

Table IV: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Exp. 6 (%)	Mean ± SEM	Significance	
								$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100	100	100 ± 0.00		
1	98.86	40.10	100.14	74.05	71	94.03	87.61 ± 6.26	N S	NS
2	56.36	44.80	84.96	76.61	70.20	89.04	75.43 ± 5.78	NS	S
3	53.23	25.44	68.58	78.58	64.66	87.25	70.46 ± 5.83	S	S
4	41.55	39.59	62.88	71.84	58.81	91.74	54.41 ± 6.53	S	S

The plot of data of total PAR of blood lymphocyte proteins also shows a negative correlation as in other tissues. In the 4th week of DMN exposure the total PAR reduces to 45 % as compared to the control (Fig. 18).

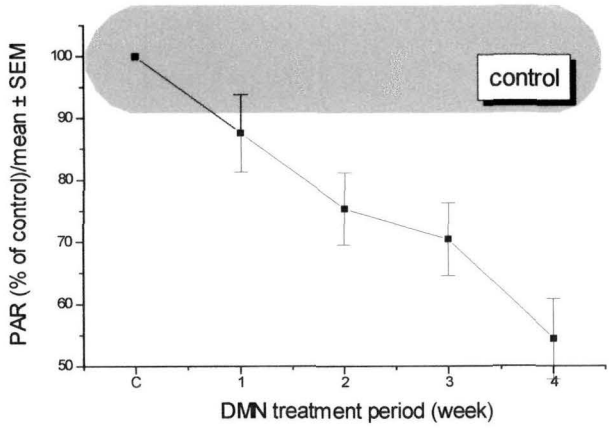


Figure 18. Relationship between total PAR of cellular proteins of blood lymphocytes in control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN treatment in week.

3.9.2. Influence of DMN + 3-AB treatment on total PAR

I) Liver:

The band intensities were quantified densitometrically (§ 2.26.) to obtain 'Adj Volume OD x mm^2' which represents the mean intensity or pixel density of bands. Results of all immunodetection experiments for total PAR of liver proteins in control and DMN + 3-AB treated mice (§ 2.18.) were pooled. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table V). The statistical significance of difference between control and DMN + 3-AB exposed groups was calculated at $P \leq 0.01$ and $P \leq 0.05$. The result is shown in Table V below.

Table V: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the data are significantly different.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Mean ± SEM	Significance	
							$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100	100 ± 0.00		
1	83.55	71.57	68.25	74.45	79.44	75.45 ± 2.73	S	S
2	62.31	52.88	77.18	55.75	70.05	63.63 ± 4.49	S	S
3	32.06	39.17	59.83	43.77	57.59	46.48 ± 5.34	S	S
4	29.82	35.45	60.13	38.49	69.46	46.67 ± 7.67	S	S

As can be seen from the plot of data in Fig. 19, there is a clear negative correlation between total PAR in liver and the period of DMN + 3-AB treatment. In the 4th week of treatment there was 55 % reduction in the level of PAR in comparison with the control.

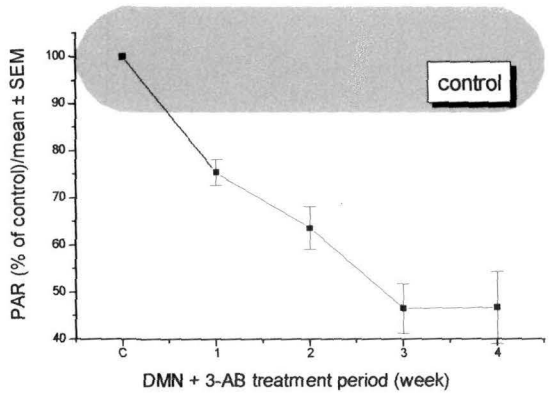


Figure 19. Relationship between total PAR of cellular proteins of liver of control and DMN + 3-AB exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN+ 3-AB treatment in week.

II) Spleen:

Results of immunodetection for total PAR of proteins of SC in control and DMN + 3-AB treated samples (§ 2.18.) were quantified densitometrically (§ 2.26.) as done in case of liver. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table VI).

Table VI: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	70.81	78.84	92.32	90.02	82.99 ± 5.01	N S	S
2	46.96	68.04	59.00	70.77	61.19 ± 5.36	S	S
3	39.85	29.86	26.00	45.29	35.25 ± 4.44	S	S
4	25.88	23.93	33.29	30.96	28.51 ± 2.17	S	S

The plot of data is shown in Fig. 20. It revealed that there was again almost a straight

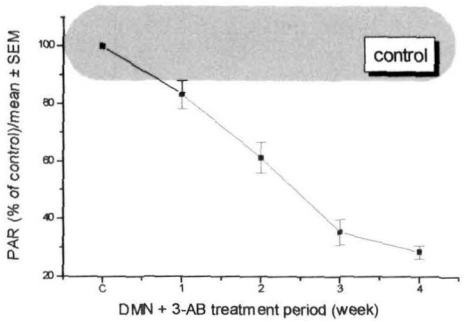


Figure 20. Relationship between PAR of total cellular proteins of spleen cells in control and DMN + 3-AB exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN + 3-AB treatment in week.

but a negative correlation between the level of PAR in SC and the period of DMN + 3-AB treatment. In the 4^{th} week of treatment the reduction was up to 70 % of the control.

III) BMC:

Similar strategy was employed for the densitometric quantification of total PAR of BMC proteins in control and DMN + 3-AB exposed mice. The results are shown in Table VII. The statistical significance of the data was calculated at $P \leq 0.01$ and $P \leq 0.05$.

Table VII: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	59.33	79.21	65.55	79.32	70.85 ± 5.02	N S	S
2	33.81	36.88	44.53	45.66	40.22 ± 2.89	S	S
3	30.70	29.00	25.32	30.66	28.92 ± 1.26	S	S
4	20.42	13.45	16.32	21.76	18.11 ± 1.87	S	S

Upon plotting the data in a graph again an almost straight but a negative correlation between total PAR in BMC proteins and the period of DMN + 3-AB treatment was found. In the 4 weeks treated group the reduction was more than 80 % of the control.

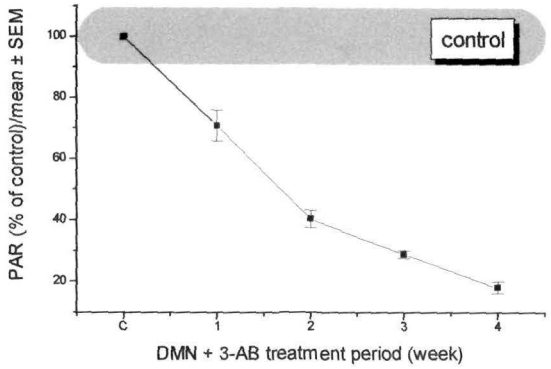


Figure 21. Relationship between total PAR of cellular proteins of BMC in control and DMN + 3-AB exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN+ 3-AB treatment in week.

IV) Blood lymphocytes:

The total PAR of blood lymphocyte proteins of control and DMN + 3-AB treated mice (§ 2.18.) were similarly quantified densitometrically (§ 2.26.). The results of individual experiments have been shown in Table VIII.

Table VIII: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	45.14	59.41	48.39	47.12	50.01 ± 3.20	S	S
2	37.48	45.61	41.95	43.61	42.16 ± 1.73	S	S
3	27.34	42.94	32.10	39.54	35.48 ± 3.53	NS	S
4	21.70	31.51	31.61	28.75	28.39 ± 2.32	S	S

The plot of the data of total PAR of blood lymphocyte proteins also shows a negative correlation as in other tissues. In the 4th week of DMN + 3-AB exposure the total PAR reduces to 30 % as compared with the control (Fig. 22).

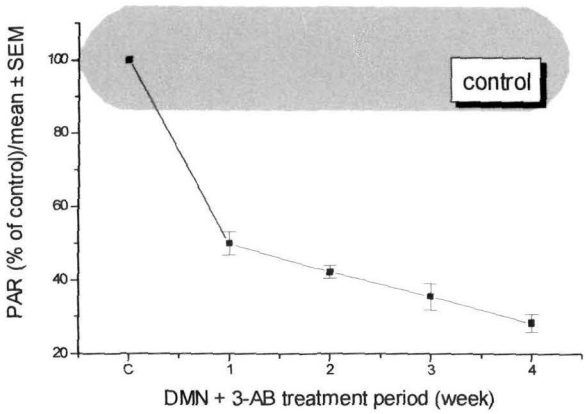


Figure 22. Relationship between total PAR of cellular proteins of blood lymphocyte proteins of in control and DMN + 3-AB exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN + 3-AB treatment in week.

3.9.3. Influence of DMN on total PAR of histones

I) Liver:

The band intensities were quantified densitometrically (§ 2.26.) to obtain 'Adj Volume OD x mm^2' which represents the mean intensity or pixel density of bands. Results of all immunodetection experiments for PAR of liver histones in control and DMN treated mice (§ 2.17.) were pooled. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table IX). The statistical significance of difference between control and DMN exposed groups was calculated at $P \leq 0.01$ and $P \leq 0.05$. The result is shown in table IX below. Fig. 23 shows that there is a negative correlation between the level of PAR of liver histones and the period of DMN treatment.

Table IX: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Mean ± SEM	Significance	
							$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100	100 ± 0.00		
1	63.64	46.34	66.87	58.67	99.89	67.08 ± 8.91	NS	S
2	50.67	89.85	71.81	81.23	98.66	78.44 ± 8.25	NS	NS
3	61.98	80.32	50.37	55.63	91.75	68.01 ± 7.79	NS	S
4	60.93	35.13	37.07	40.83	66.86	48.16 ± 6.55	S	S

In the 4^{th} week of treatment there is more than 50 % reduction in the level of PAR when compared with the control (Fig. 23).

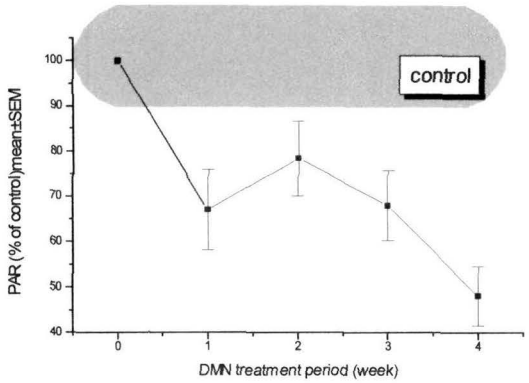


Figure 23. Relationship between PAR of liver histones in control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN treatment.

II) Spleen:

Results of immunodetection for PAR of histones of SC in control and DMN exposed mice (§ 2.17.) were quantified densitometrically (§ 2.26.) as done in case of liver histones. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table X).

Table X: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						P≤ 0.01	P≤ 0.05
0	100	100	100	100	100 ± 0.00		
1	73.07	58.97	51.31	56.88	60.05 ± 4.62	S	S
2	46.14	58.50	60.07	61.89	56.65 ± 3.57	S	S
3	38.75	30.02	40.56	35.87	36.30 ± 2.30	S	S
4	24.21	24.86	26.59	30.91	26.60 ± 1.50	S	S

Upon plotting the data in a graph again a negative correlation between the level of PAR of spleen histones and the period of DMN treatment was found. In the 4th week of treatment the reduction was up to 70 % of the control (Fig. 24).

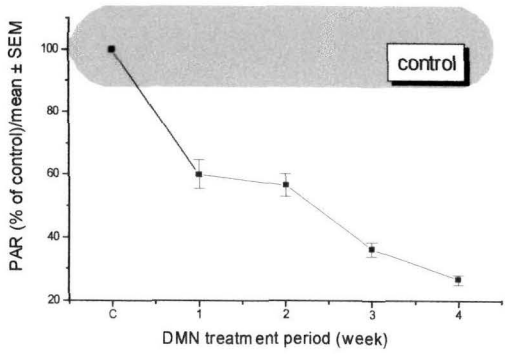


Figure 24. Relationship between PAR of histones of spleen cells in control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN treatment.

III) Blood lymphocytes:

The PAR of histones of blood lymphocytes of control and DMN exposed mice (§ 2.17.) were similarly quantified densitometrically (§ 2.26.). The results of individual experiments have been shown in Table XI. The plot of data of blood lymphocyte histones shows almost a straight but a negative correlation between the level of PAR and the period of DMN treatment. In the 4th week of treatment the reduction was more than 60 % when compared with the control (Fig. 25).

Table XI: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Exp. 6 (%)	Exp. 7 (%)	Mean ± SEM	Significance	
									P≤ 0.01	P≤0.05
0	100	100	100	100	100	100	100	100 ± 0.00		
1	91.51	71.07	83.20	91.01	89.21	67.76	94.76	85.75 ± 3.87	S	S
2	62.83	52.89	47.38	66.96	64.45	66.68	94.13	64.50 ± 4.88	S	S
3	57.00	49.15	36.44	65.60	52.32	39.97	75.51	51.96 ± 4.83	S	S
4	48.13	41.79	30.96	44.25	33.84	36.39	45.15	39.61 ± 2.14	S	S

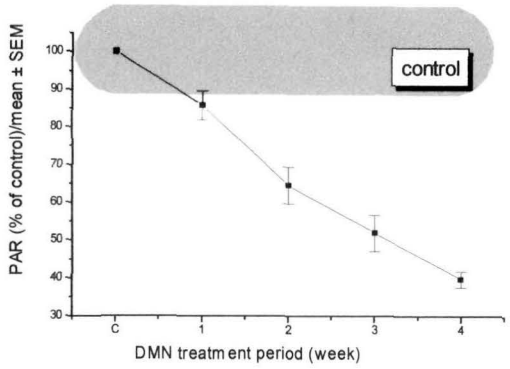


Figure 25. Relationship between PAR of blood lymphocyte histones in control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of DMN treatment.

3.9.4. Influence of AEBN on total PAR

I) Spleen:

The band intensities were quantified densitometrically (§ 2.26.) to obtain 'Adj Volume OD x mm^2' which represents the mean intensity or pixel density of bands. Results of all immunodetection experiments for total PAR of SC proteins in control and AEBN treated mice (§ 2.18.) were pooled. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table XII). The statistical significance of difference between control and AEBN exposed groups was calculated at $P \leq 0.01$ and $P \leq 0.05$.

Table XII: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	93.11	99.42	91.92	99.80	96.06 ± 2.06	NS	NS
2	53.22	50.05	60.34	58.84	55.61 ± 2.40	S	S
3	52.71	47.99	45.43	42.54	47.16 ± 2.15	S	S
4	38.46	32.96	29.27	39.47	35.04 ± 2.39	S	S

The plot of data in Fig. 26 shows a negative correlation between total PAR of SC proteins and the period of AEBN treatment was found. In the 4^{th} week of treatment there was up to 70 % reduction PAR level when compared with the control.

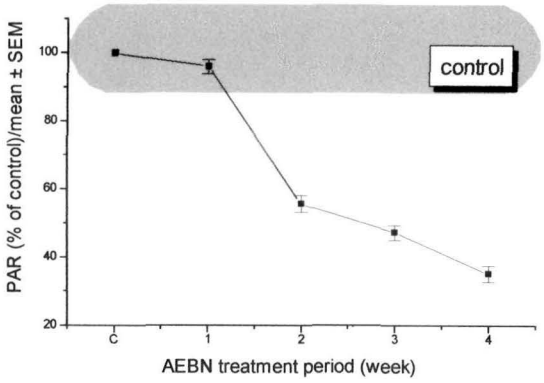


Figure 26. Relationship between total PAR of cellular proteins of spleen cells proteins in control and AEBN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment.

II) BMC:

Following similar strategy, the results of immunodetection for total PAR of proteins of BMC in control and AEBN treated samples (§ 2.18.) were quantified densitometrically (§ 2.26.). The data have been expressed as per cent (%) of control in term of mean ± SEM. The results are shown in Table XIII.

Table XIII: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	40.38	42.89	45.76	40.87	42.47 ± 1.22	S	S
2	39.36	40.86	42.45	38.85	40.38 ± 0.81	S	S
3	39.18	38.89	39.96	40.15	39.54 ± 0.30	S	S
4	23.18	25.16	26.66	23.63	24.65 ± 0.79	S	S

Upon plotting the data in a graph, a negative correlation between total PAR of BMC proteins and the period of AEBN treatment was observed. Up to 80 % reduction in the 4th week of treatment was obtained in comparison to the control (Fig. 27).

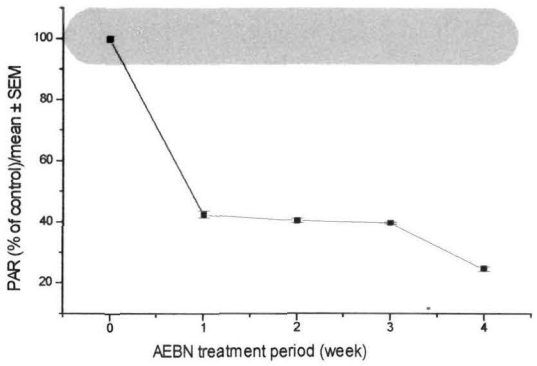


Figure 27. Relationship between total PAR of cellular proteins of BMC in control and AEBN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment.

III) Blood lymphocytes:

Densitometric quantification (§ 2.26.) for various immunodetection experiments for the total PAR of blood lymphocyte proteins of control and AEBN exposed mice (§ 2.18.) was done similarly. The results are summed up in Table XIV below. The plot of data in Fig. 28 also shows a negative correlation between total PAR of blood lymphocyte proteins and the period of AEBN treatment as observed in other tissues.

Table XIV: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Exp. 6 (%)	Mean ± SEM	Significance	
								P≤ 0.01	P≤ 0.05
0	100	100	100	100	100	100	100 ± 0.00		
1	86.06	87.82	81.89	82.12	79.07	83.92	83.48 ± 1.28	S	S
2	65.76	66.00	77.31	80.31	60.35	59.48	68.19 ± 3.55	S	S
3	40.31	62.73	74.29	68.09	41.87	45.37	55.44 ± 6.00	S	S
4	33.69	53.18	64.10	49.09	39.15	30.81	45.00 ± 5.20	S	S

The reduction in the 4th week of treatment was up to 55 % of the control (Fig. 28).

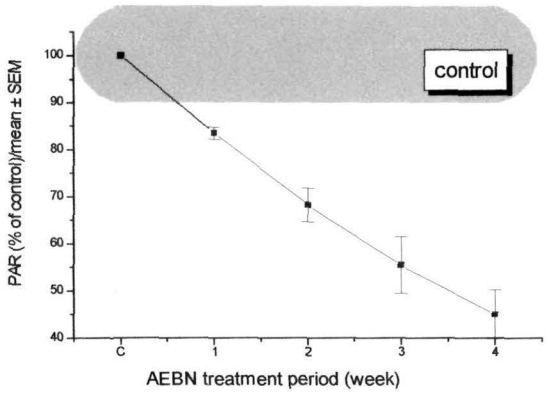


Figure 28. Relationship between total PAR of cellular proteins of blood lymphocytes in control and AEBN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected slots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment.

3.10. PROTEIN PROFILE OF LIVER, BLOOD LYMPHOCYTES, SC OR BMC AFTER DMN TREATMENT

WH were prepared from liver, blood lymphocytes, SC or BMC of normal and DMN treated mice (§ 2.16.). SDS-PAGE was carried out to obtain the protein profiles of different WH (§ 2.19.). The gels were stained with coomassie brilliant blue (§ 2.19.2.), destained and photographed using Kodak digital camera (§ 2.19.2.).

The protein profile of various tissues of normal and DMN treated mice does not show any noticeable change between the profile of proteins of DMN-exposed tissues and control. However, there was some variation in the level of expression of various proteins as seen in Fig. 29.

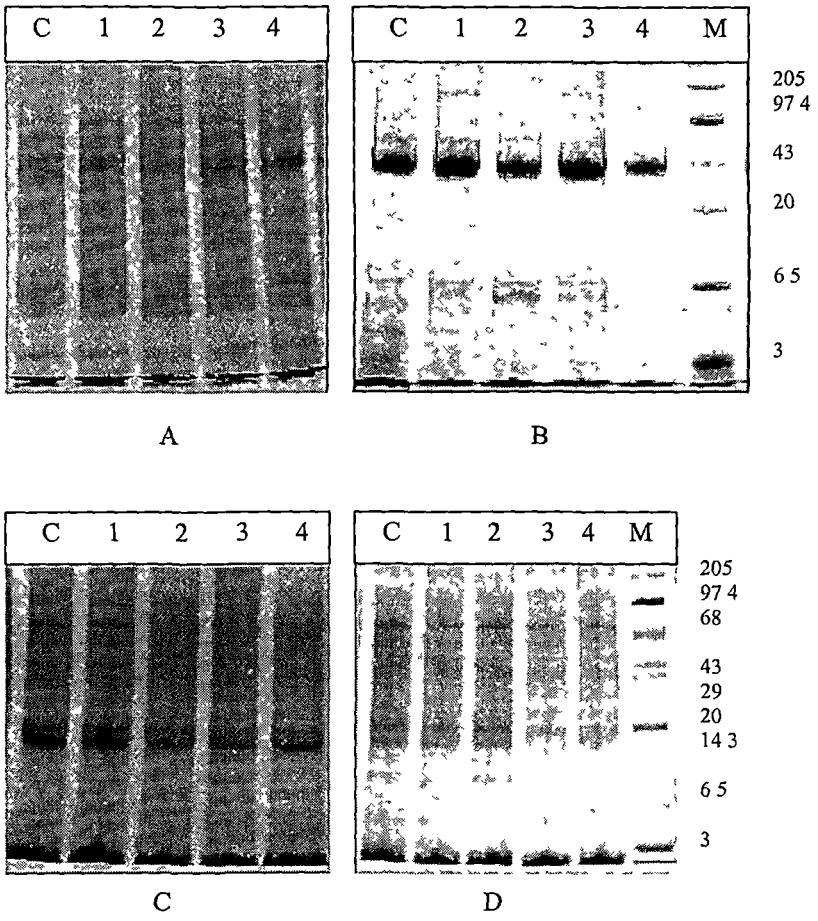


Figure 29. SDS-PAGE protein profiles of whole homogenate of different tissues of mice stained with coomassie brilliant blue. A - liver, B - blood lymphocytes, C – spleen cells and D - bone marrow cells. C - control, 1, 2, 3 and 4 (DMN treatment period in week respectively), M - molecular weight marker in kDa.

3.11. PROTEIN PROFILE OF LIVER, BLOOD LYMPHOCYTES, SC OR BMC AFTER DMN + 3-AB TREATMENT

WH were prepared from liver, blood lymphocytes, SC or BMC of normal and DMN + 3-AB treated mice (§ 2.17.). SDS-PAGE was carried out to obtain the protein profiles of different samples (§ 2.19.). The gels were stained with coomassie brilliant blue (§ 2.19.2.), destained and photographed using Kodak digital camera (§ 2.19.2.).

No noticeable change was observed between the protein profile of DMN + 3-AB-exposed tissues and control. However, some variation in the level of expression of some proteins can be seen (Fig. 30).

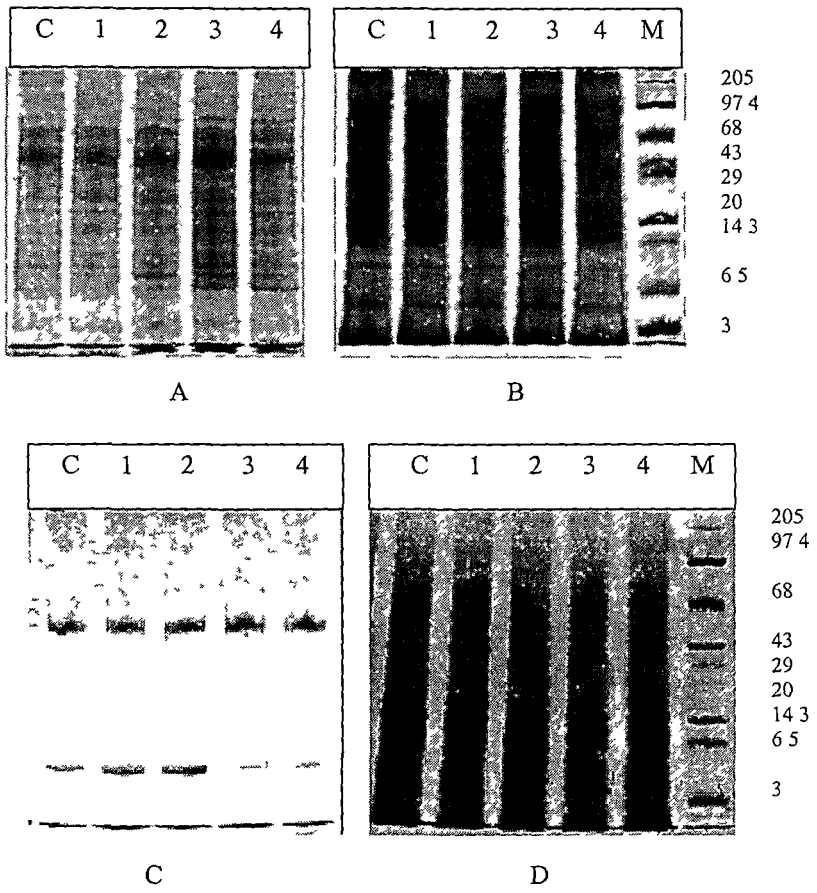


Figure 30 SDS-PAGE protein profiles of whole homogenate of different tissues of mice stained with coomassie brilliant blue. A - bone marrow cells, B - liver, C - blood lymphocytes and D – spleen cells C - control, 1, 2, 3 and 4 (DMN + 3-AB treatment period in week respectively), M - molecular weight marker in kDa

3.12. HISTONE PROTEIN PROFILES OF BLOOD LYMPHOCYTES, LIVER OR SC AFTER DMN TREATMENT

Histone proteins were isolated from blood lymphocytes, liver and SC of normal and DMN exposed mice (§ 2.17.). SDS-PAGE was carried out to obtain the histone profiles of different samples (§ 2.19.). The gels were stained with coomassie brilliant blue (§ 2.19.2.), destained and photographed using Kodak digital camera (§ 2.19.2.).

The SDS-PAGE profiles of histone proteins in Fig. 31 does not show noticeable change between the profile of DMN treated tissues and control.

3.13. PROTEIN PROFILE OF SC, BLOOD LYMPHOCYTES OR BMC AFTER AEBN TREATMENT

WH were prepared from blood lymphocytes, SC or BMC of normal and AEBN treated mice (§ 2.16.). SDS-PAGE was carried out to obtain the protein profiles of different samples (§ 2.19.). The gels were stained with coomassie brilliant blue (§ 2.19.2.), destained and photographed using Kodak digital camera (§ 2.19.2.).

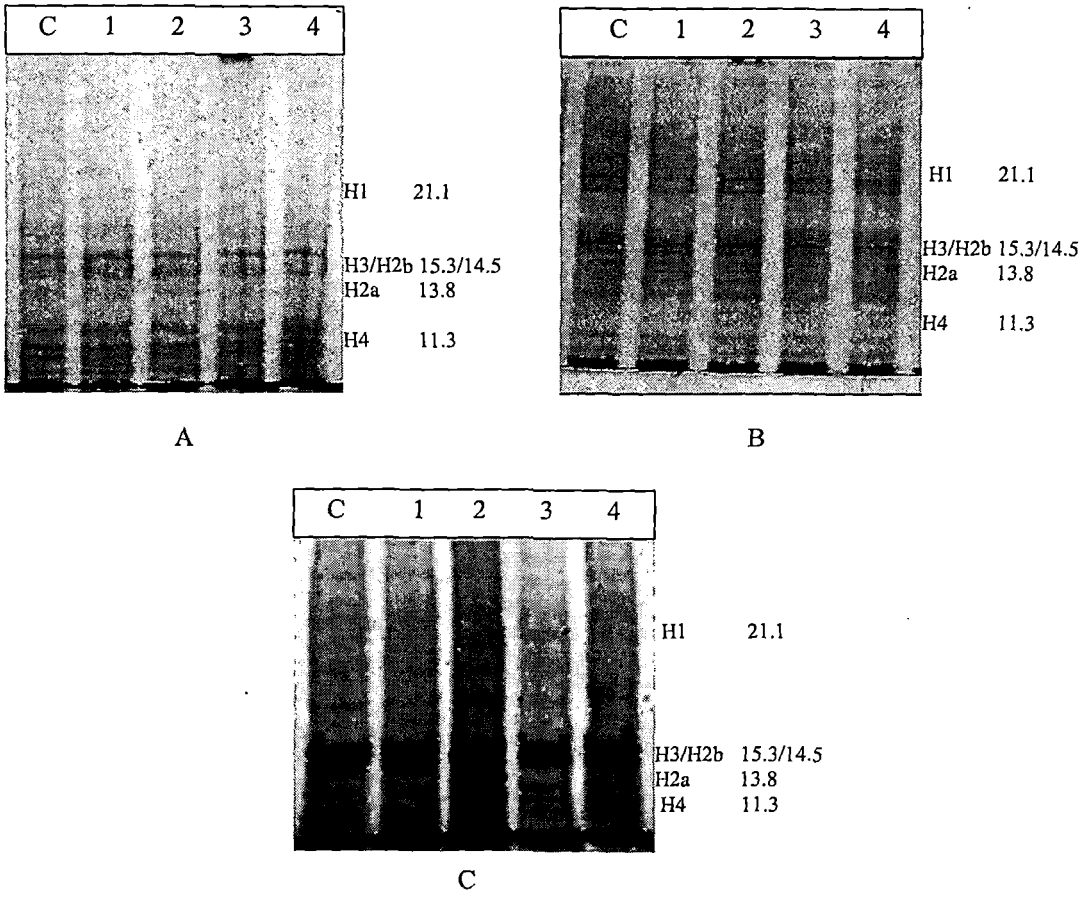


Figure 31. SDS-PAGE profiles of histone proteins of different tissues of mice stained with coomassie brilliant blue. A - blood lymphocytes and B - liver histones were subjected to SDS-PAGE at 150 V constant for 1 h; and C - histones of spleen cells was subjected to SDS-PAGE at 200 V constant for 45 min. C - control, 1, 2, 3 and 4 (DMN treatment period in week respectively). M - molecular weight marker in kDa.

The protein profile of control and AEBN-exposed tissues of mice in Fig. 32 below does not show any noticeable change. However, some variation in the level of expression of some proteins can be observed.

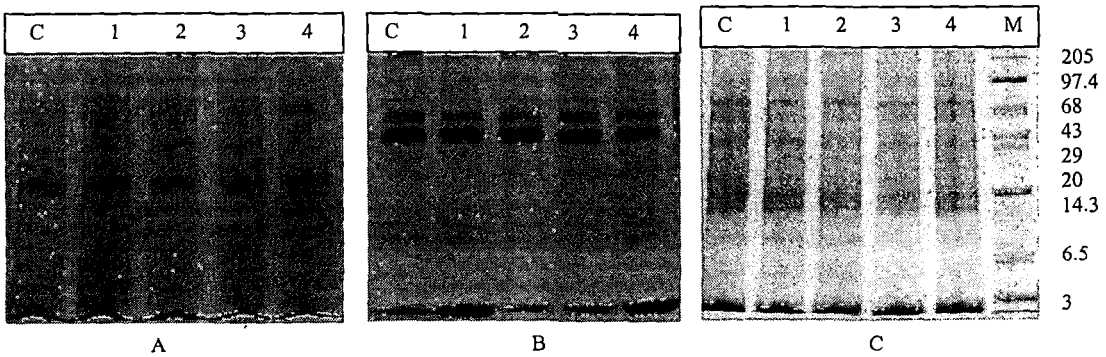


Figure 32. SDS-PAGE protein profile of whole homogenate of different tissues of mice stained with coomassie brilliant blue. A – spleen cells, B - blood lymphocytes, and C - bone marrow cell. C - *control, 1, 2, 3 and 4 (AEBN treatment period in week, respectively). M - molecular weight marker in* kDa.

3.14. INFLUENCE OF DMN ON PAR OF INDIVIDUAL CELLULAR PROTEINS

WH were prepared from SC, liver, blood lymphocytes or BMC of normal and DMN treated mice (§ 2.16.). The proteins resolved by SDS-PAGE gels (§ 2.19.) were transferred to PVDF membrane by Western blotting (§ 2.20.). In Fig. 33 (I) a Western blot membrane, which was immunoprobed for PAR (§ 2.21.) and (II) a replica of it stained with India ink for total protein content (§ 2.22.).

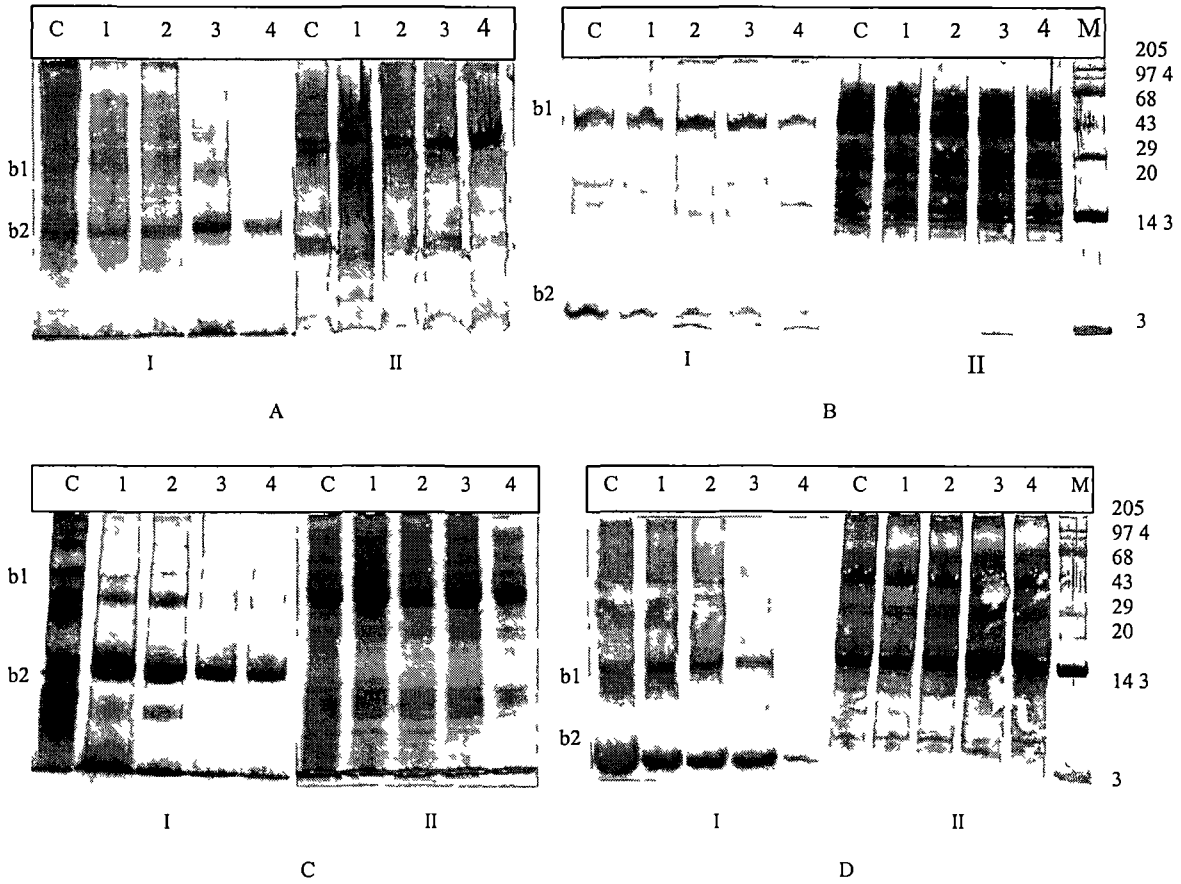


Figure 33 Whole homogenate of different tissues after it was subjected to SDS-PAGE and Western blotting. One of the membranes was stained by India ink for total protein content (II) and a replica of it was immunoprobed for PAR (I). A – spleen cells, B - liver and C – blood lymphocytes and D - bone marrow cell. C - control, 1, 2, 3 and 4 (DMN treatment period in week, respectively). M - molecular weight marker in kDa. Band 1 and 2, which have been further analyzed (§ 3 18 1) are marked as b1 and b2, respectively on the left side.

In Fig. 33 above, progressive decrease in intensity of bands, which is directly related to the level of PAR of proteins in the tissues in DMN treatment groups when compared with the control (I). The level of reduction of PAR of various individual cellular proteins was quite significant in the 3rd and 4th week of treatment in almost all tissues. There was no noticeable variation in the total protein content during the treatment period when compared with the control (II).

3.15. INFLUENCE OF DMN + 3-AB ON PAR OF INDIVIDUAL CELLULAR PROTEINS

SDS-PAGE was carried out (§ 2.19.) for whole homogenate of SC, liver, blood lymphocytes or BMC from normal and DMN + 3-AB exposed mice (§ 2.16.). The proteins on the gels were then transferred to PVDF membrane by Western blotting (§ 2.20.). Fig. 34 (I) shows a Western blot immunoprobed for PAR (§ 2.21.) and (II) shows a replica of it India ink stained for total protein content (§ 2.22.).

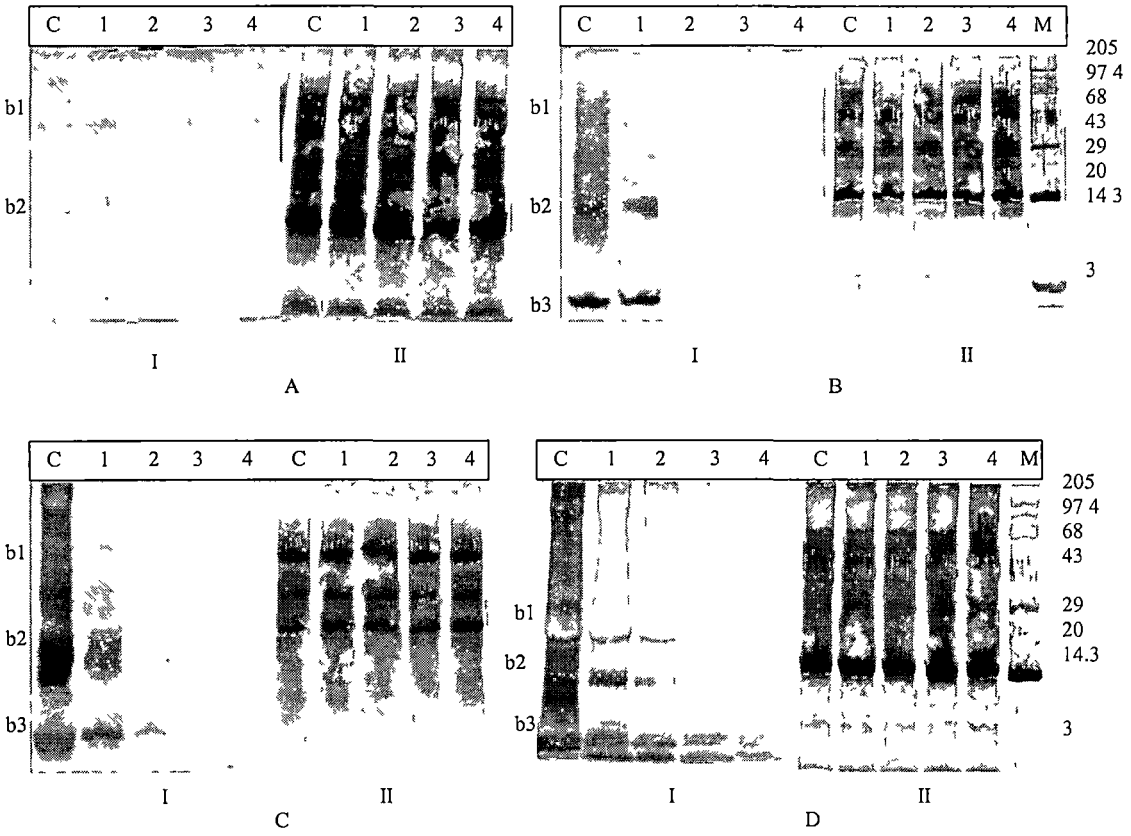


Figure 34. Whole homogenate of different tissues after it was subjected to SDS-PAGE and Western blotting. One of the membranes was India ink stained for total protein content (II) and a replica of it was immunoprobed for PAR (I). A - liver, B - bone marrow cell, C - spleen cells and D - blood lymphocytes. C - control, 1, 2, 3 and 4 (DMN + 3-AB treatment period in week, respectively). M - molecular weight marker in kDa. Band 1, 2 and 3, which have been further analyzed (§ 3.18.2.) are marked as b1, b2 and b3, respectively on the left side.

Upon DMN + 3-AB treatment, more intense and progressive reduction in band intensity was observed (Fig. 34 (I)). There was significant reduction in the level of PAR of many proteins in the 2nd, 3rd and 4th week of treatment in almost all tissues. Total protein Western blotted on the membrane was essentially equal (Fig. 34 (II)).

3.16. INFLUENCE OF DMN ON PAR OF INDIVIDUAL HISTONE PROTEINS

Histone proteins were isolated from SC, liver or blood lymphocytes of normal and DMN treated mice (§ 2.17.). SDS-PAGE was carried out (§ 2.19.) and the proteins were transferred to PVDF membrane by Western blotting (§ 2.20.). Fig. 35 (I) below

shows immunoprobed Western blot for PAR (§ 2.21.) and (II) shows a replica of it that has been stained with India ink for total protein content (§ 2.22.).

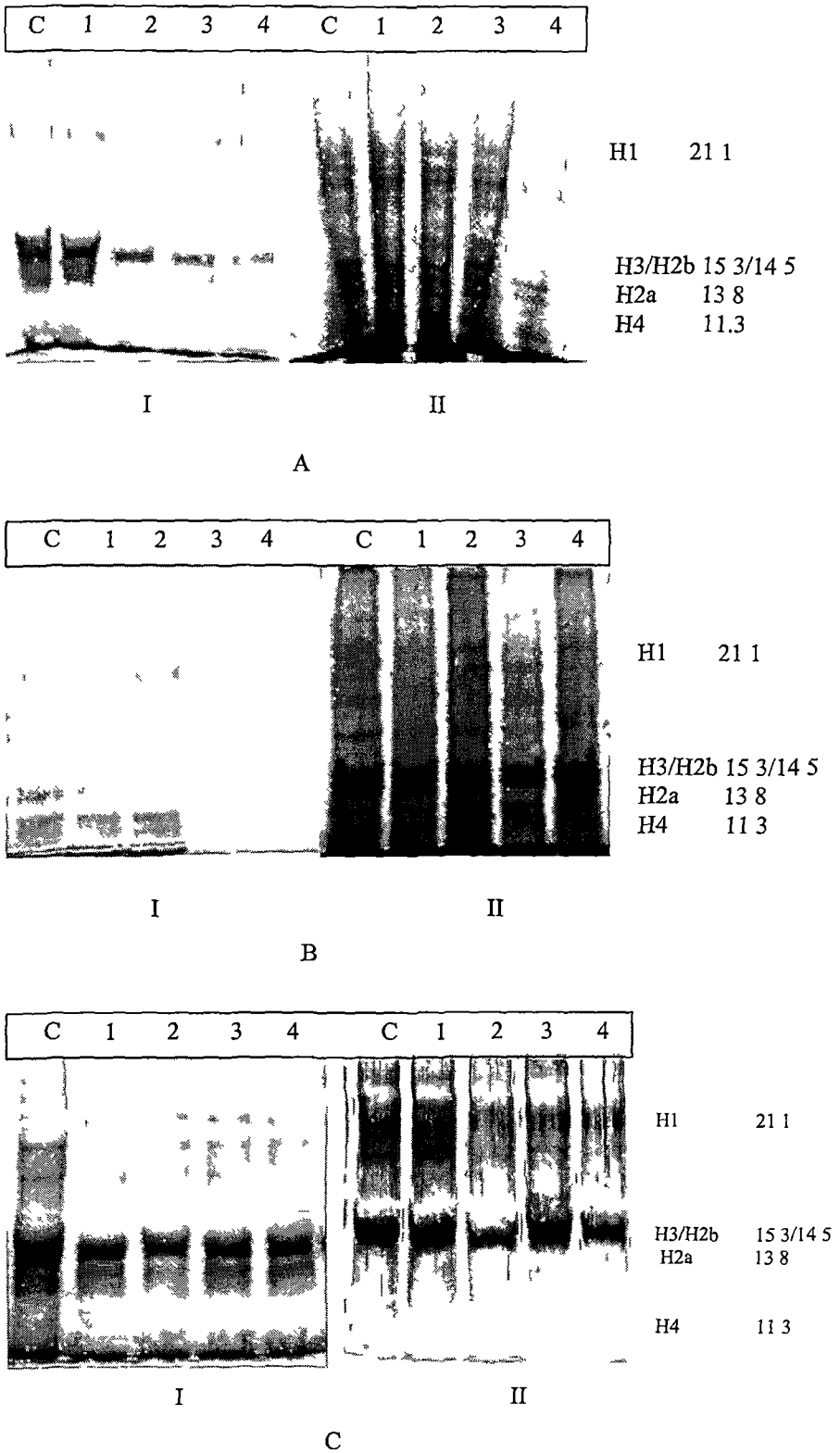


Figure 35 Histone proteins of mice after it were subjected to SDS-PAGE A - liver and B - spleen cells were run at 200 V constant for 45 min C - blood lymphocytes samples were run at 150 V constant for 1 h One of the Western blotted membranes was stained by India ink for total protein content (II) and a replica of it was immunoprobed for PAR (I) C - control, 1, 2, 3 and 4 (DMN treatment period in week, respectively) M - molecular weight marker in kDa.

A reduction in the band intensity, which represents a fall in the level of PAR of various histones of different tissues, was observed in the DMN treated groups when compared

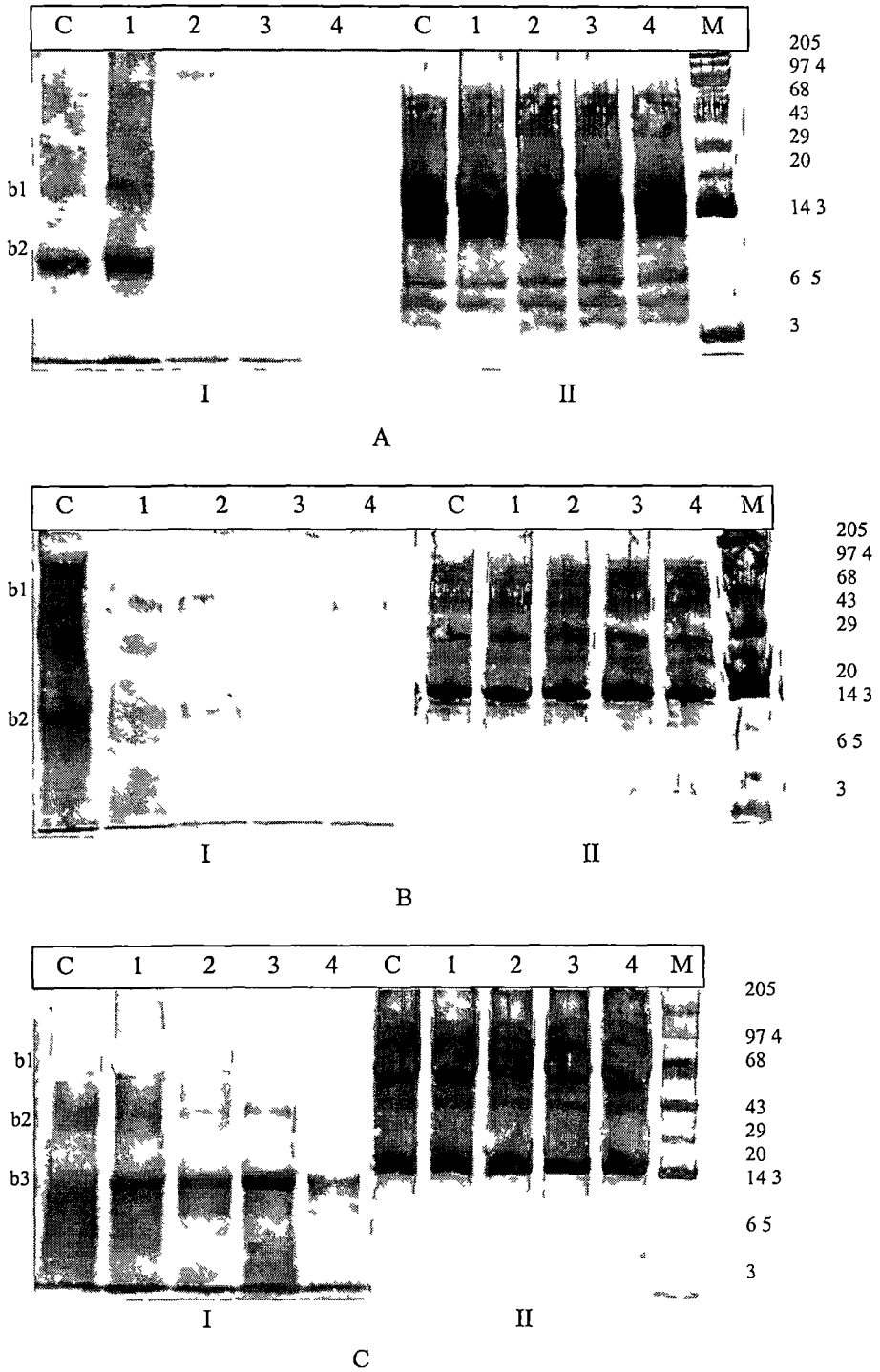


Figure 36 Whole homogenate of different tissues of mice after it was subjected to SDS-PAGE and Western blotting One of the Western blots was stained by India ink for total protein content (II) and a replica of it was immunoprobed for PAR (I) A – spleen cells, B - bone marrow cell and C – blood lymphocytes C - control, 1, 2, 3 and 4 (AEBN treatment period in week, respectively) M - molecular weight marker in kDa Band 1, 2 and 3, which have been further analyzed (§ 3 18 4) are marked as b1, b2 and b3, respectively on the left side

with the control (Fig. 35 (I)). Significant reduction in the level of PAR was observed in the 3rd and 4th week of treatment in almost all tissues. No noticeable variation in the total protein content was seen in control and treated groups (II).

3.17. INFLUENCE OF AEBN ON PAR OF INDIVIDUAL CELLULAR PROTEINS

WH were prepared from SC, blood lymphocytes or BMC of normal and AEBN treated mice (§ 2.16.). SDS-PAGE was carried (§ 2.19.) and the proteins were transferred to PVDF membrane by Western blotting (§ 2.20.). Fig. 36 (I) shows a Western blot immunoprobed (§ 2.21.) for PAR and a replica of it was stained with India ink (II) for total protein content (§ 2.22.). In Fig. 36 above, a progressive decrease in the intensities of different bands was observed in AEBN-exposed groups when compared with the control (Fig. 36 (I)). Significant reduction in the level of PAR was observed in the 2nd, 3rd and 4th week of treatment in all cases. No noticeable variation in the total protein content was seen in control and the AEBN treated groups (II).

3.18. QUANTITATIVE ANALYSIS OF PAR OF INDIVIDUAL CELLULAR PROTEINS

3.18.1. Influence of DMN

I) Liver:

Band 1: The intensity of selected band, b1 (Fig. 33 B), was quantified densitometrically (§ 2.26.) to obtain 'Adj Volume OD x mm^2' which represents the mean intensity or pixel density of different bands. Results of all Western blot (§ 2.20.) immunodetection experiments for PAR of individual proteins in control and DMN treated mice (§ 2.21.) were pooled. The data has been expressed as per cent (%) of control in term of mean $\pm$ SEM (Table XV). The statistical significance of difference between control and DMN treated groups was calculated at $P \leq 0.01$ and $P \leq 0.05$.

Table XV: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp 1 (%)	Exp 2 (%)	Exp 3 (%)	Exp 4 (%)	Mean $\pm$ SEM	Significance	
						P ≤0 01	P ≤ 0 05
0	100	100	100	100	100 $\pm$ 0 00		
1	91 36	93 63	89 05	91 77	91 45 $\pm$ 0 94	S	S
2	85 95	75 03	70 56	85 21	79 18 $\pm$ 3 80	NS	S
3	80 09	89 57	72 15	86 76	82 14 $\pm$ 3 87	NS	S
4	49 65	48 89	45 06	44 18	46 94 $\pm$ 1 36	S	S

Band 2: Steps similar to band 1 were followed for band 2 (b2, Fig. 33 B). The result is shown in table XVI.

As can be seen in the Fig. 37 below there is a negative correlation between the level of PAR of liver and the period of DMN treatment. In the 4th week of treatment there

Table XVI: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	52.68	66.51	55.86	42.12	54.29 ± 5.02	S	S
2	45.09	63.23	48.56	39.30	49.04 ± 5.09	S	S
3	31.65	42.49	44.56	30.54	37.31 ± 3.62	S	S
4	26.15	30.67	29.86	25.78	28.12 ± 1.25	S	S

was up to 55 % reduction PAR level of band 1 and 75 % reduction in band 2 when compared with the control (Fig. 37).

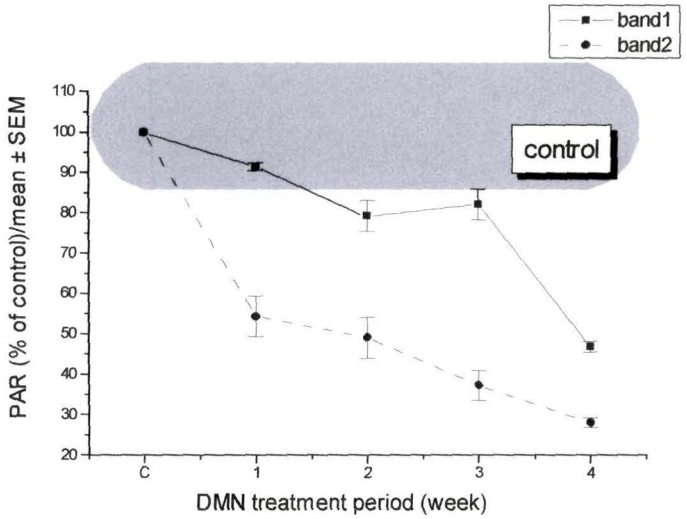


Figure 37. Relationship between PAR of cellular protein bands b1 and b2 of liver cells in control and DMN-exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1- protein of molecular weight 43 kDa and Band 2 - protein of molecular weight < 7 kDa (Fig. 33 B).

II) Spleen:

Band 1: Results of immunodetection for PAR of cellular protein band b1 (Fig. 33 A) of SC in control and DMN treated samples (§ 2.21.) were quantified densitometrically

Table XVII: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	76.58	85.23	65.05	94.65	80.37 ± 6.30	NS	N S
2	66.34	52.70	44.15	57.04	55.05 ± 4.61	S	S
3	58.84	50.18	33.72	54.37	49.27 ± 5.47	S	S
4	42.05	23.54	30.14	33.60	32.33 ± 3.85	S	S

(§ 2.26.) as done in case of liver. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table XVII).

Band 2: Similarly, densitometric quantification (§ 2.26.) was done for band 2 (b2, Fig. 33 A) also. The result is shown in Table XVIII.

Table XVIII: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	79.55	62.20	46.29	47.85	58.97 ± 7.73	NS	S
2	68.80	64.46	33.73	33.21	50.05 ± 9.61	NS	S
3	58.15	58.63	33.50	28.93	44.80 ± 7.90	S	S
4	26.16	28.31	29.55	24.45	27.11 ± 1.31	S	S

Here too, a negative correlation between the level of PAR in liver proteins and the period of DMN treatment was observed. The reduction PAR level of band 1 and band 2 in the 4th week of treatment are over 65 % and 70 % of control, respectively (Fig. 38).

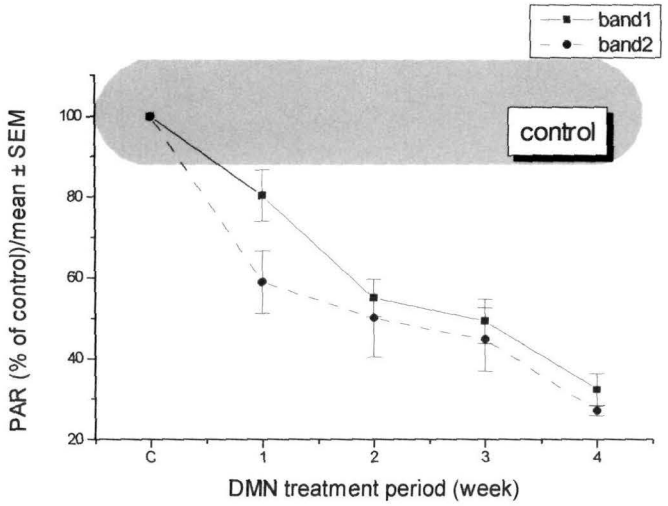


Figure 38. Relationship between PAR of cellular protein bands b1 and b2 of spleen cells of control and DMN treated mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1- protein of molecular weight 20 kDa (likely to be H1) and Band 2 - protein of molecular weight 14.3 kDa (likely to be H2b) (Fig. 33 A).

III) BMC:

Band 1: Similar strategy was used for densitometric quantification (§ 2.26.) of the PAR of cellular protein band b1 (Fig. 33 D) of BMC in control and DMN exposed mice (§ 2.16.). The result is shown in Table XIX. The data has been expressed as per cent (%) of control in term of mean ± SEM. The statistical significance of the data was calculated at $P \leq 0.01$ and $P \leq 0.05$.

Table XIX: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	97.08	82.93	71.65	83.88 ± 7.35	NS	N S
2	63.56	59.34	66.48	63.12 ± 2.07	S	S
3	49.15	47.09	40.25	45.76 ± 2.78	S	S
4	21.09	19.15	21.33	20.50 ± 0.69	S	S

Band 2: Densitometric quantification (§ 2.26.) for band 2, b2 (Fig. 33 D) was carried out as done for band 1. The statistical significance of the data was calculated similarly. The result is shown in Table XX below.

Table XX: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	70.45	76.47	81.01	75.97 ± 3.05	NS	S
2	64.12	67.03	62.38	63.17 ± 0.50	S	S
3	53.98	53.76	63.76	57.16 ± 3.29	S	S
4	15.41	17.95	17.78	17.04 ± 0.81	S	S

Upon plotting the data in a graph, again a negative correlation between the level of PAR of b1 and b2 protein bands of BMC and the period of DMN treatment can be observed (Fig. 39). In the 4th week of treatment there was about 80 % reduction in PAR level of band 1 and more than 80 % reduction in band 2 in comparison to control.

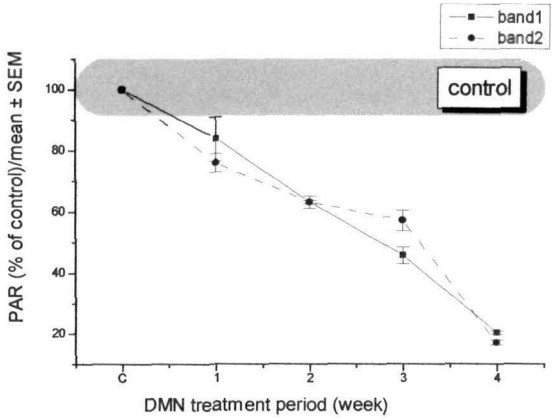


Figure 39. Relationship between PAR of cellular protein bands b1 and b2 of BMC in control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1- protein of molecular weight 14.3 kDa (likely to be H2b) and Band 2 - protein of molecular weight < 7 kDa (Fig.33 D).

IV) Blood lymphocytes:

Band 1: The PAR of protein band b1 (Fig. 33 C) of blood lymphocytes of control and DMN treated mice (§ 2.16.) were quantified densitometrically (§ 2.26.) as done for other tissues. The results of individual experiments have been summed up in Table XXI.

Table XXI: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Mean ± SEM	Significance	
				$P \leq 0.01$	$P \leq 0.05$
0	100	100	100 ± 0.00		
1	39.03	69.86	54.45 ± 15.4	N S	N S
2	48.39	56.79	52.59 ± 4.20	NS	N S
3	32.56	37.55	35.05 ± 2.49	NS	S
4	16.53	29.70	23.11 ± 6.58	N S	S

Band 2: Densitometric quantification (§ 2.26.) was done for band 2, b2 (Fig. 33 C) also. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table XXII).

Table XXII: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Mean ± SEM	Significance	
				$P \leq 0.01$	$P \leq 0.05$
0	100	100	100 ± 0.00		
1	55.86	73.07	64.46 ± 8.60	N S	N S
2	65.21	51.55	58.38 ± 6.83	NS	N S
3	24.34	46.46	35.4 ± 11.1	NS	N S
4	21.03	28.70	24.86 ± 3.83	N S	S

The plot of data of PAR of protein band b1 and b2 of blood lymphocytes again reveals a negative correlation between the level of PAR and the period of DMN exposure as observed in other tissues. The reduction was almost 75 % of control in the 4^{th} week of treatment (Fig. 40).

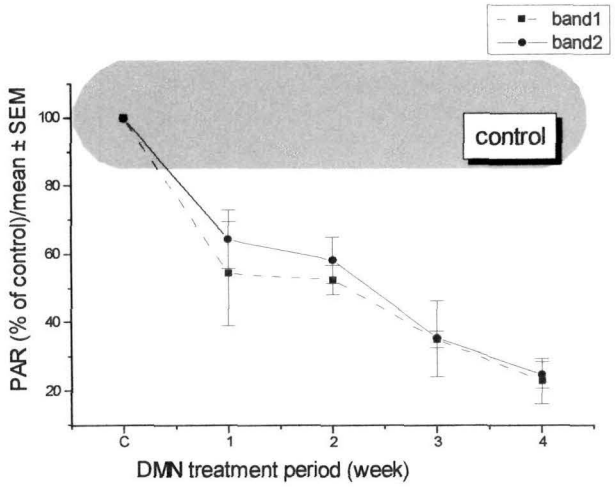


Figure 40. Relationship between PAR of cellular protein bands b1 and b2 of blood lymphocytes in control and DMN treated mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1 - protein of molecular weight 43 kDa and Band 2 - protein of molecular weight 14.3 kDa (likely to be H2b) (Fig. 33 C).

3.18.2. Influence of DMN + 3-AB

I) Liver:

Band 1: The intensity of protein band b1 (Fig. 34 A) was quantified densitometrically (§ 2.26.). Different Western blot immunodetection results of the individual liver proteins in control and DMN + 3-AB exposed mice (§ 2.21.) were combined together. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table XXIII). The statistical significance of difference between control and DMN + 3-AB exposed groups was calculated at $P \leq 0.01$ and $P \leq 0.05$. The result is shown in table XXIII below.

Table XXIII: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	78.40	62.19	75.81	71.85	72.06 ± 3.55	S	S
2	68.44	59.01	61.89	58.83	62.04 ± 2.24	S	S
3	40.59	61.82	32.33	35.65	42.59 ± 6.62	S	S
4	19.11	25.10	22.07	21.31	21.89 ± 1.23	S	S

Band 2: Densitometric quantification (§ 2.26.) was done for protein band b2 (Fig. 34 A) as in case of band 1. The result is shown in Table XXIV.

Table XXIV: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	99.33	78.28	74.32	83.97 ± 7.76	NS	NS
2	52.20	76.89	72.37	67.15 ± 7.58	NS	S
3	23.96	39.96	44.59	36.17 ± 6.24	S	S
4	41.70	20.40	46.02	36.04 ± 7.91	NS	S

The plot of data in Fig. 41 below shows that upon DMN + 3-AB treatment there was a significant reduction in the level of PAR of many proteins of liver of mice when compared with the control. There was up to 80 % reduction in the PAR level of band 1 and up to 70 % reduction in band 2 in the 4th week of treatment in comparison with the control.

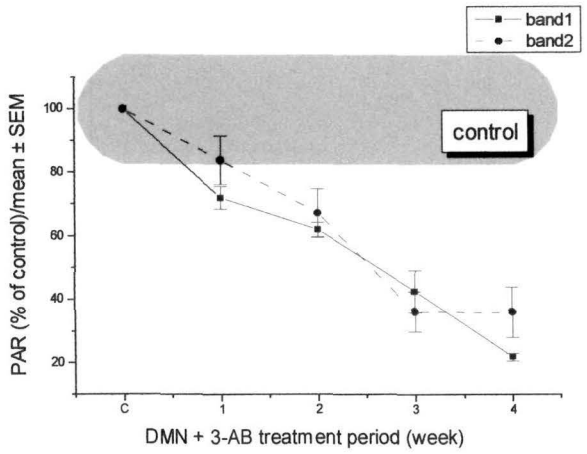


Figure 41. Relationship between PAR of cellular protein bands b1 and b2 of liver in control and DMN + 3-AB treated mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1 - protein of molecular weight 43 kDa and Band 2 - protein of molecular weight 14.3 kDa (likely to be H2b) (Fig. 34 A).

II) Spleen:

Band 1: Different results of immunodetection for PAR of protein band b1 (Fig. 34 C) of SC in control and DMN + 3-AB treated samples (§ 2.21.) were quantified densitometrically (§ 2.26.) as done for liver. The data has been expressed as per cent (%) of control in term of mean ± SEM. The results are shown in table XXV.

Table XXV: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp 1 (%)	Exp 2 (%)	Exp 3 (%)	Mean ± SEM	Significance	
					P≤0 01	P≤0 05
0	100	100	100	100 ± 0 00		
1	61 99	64 60	52 32	59 63 ± 3 37	S	S
2	54 89	54 04	55 37	54 76 ± 0 38	S	S
3	42 70	46 69	61 76	50 38 ± 5 80	NS	S
4	46 29	51 35	46 53	47 95 ± 1 54	S	S

Band 2 (group of proteins): Similarly, band 2, b2 (Fig. 34 C) was also quantified densitometrically (§ 2.26.). The results have been summed up in table XXVI.

Table XXVI: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp 1 (%)	Exp. 2 (%)	Exp 3 (%)	Mean ± SEM	Significance	
					P≤0 01	P≤0 05
0	100	100	100	100 ± 0 00		
1	55 74	47 71	58 89	54 11 ± 3 32	S	S
2	30 86	32 86	35 35	33 02 ± 1 29	S	S
3	23 16	25.67	27 15	25 32 ± 1 16	S	S
4	19 96	21 55	25 45	22 32 ± 1 63	S	S

Band 3: Band 3, b3 (Fig. 34 C) was quantified (§ 2.26.) like in other cases (Table XVII).

Table XXVII: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp 1 (%)	Exp 2 (%)	Exp 3 (%)	Mean ± SEM	Significance	
					P≤0 01	P≤0 05
0	100	100	100	100 ± 0 00		
1	61 82	66 69	70 16	66 22 ± 2 41	S	S
2	46 12	47 05	50 95	48 04 ± 1 47	S	S
3	29 85	31 34	55 59	38 92 ± 8 34	NS	S
4	27 67	25 45	34 45	29 19 ± 2 70	S	S

Upon plotting the data, it was observed that DMN + 3-AB treatment resulted in significant reduction in the level of PAR of these protein bands of SC when compared with the control. Up to 50 % reduction in the PAR level of band 1, 75 % in band 2 and about 70 % in band 3 was observed in the 4 weeks treatment groups (Fig. 42).

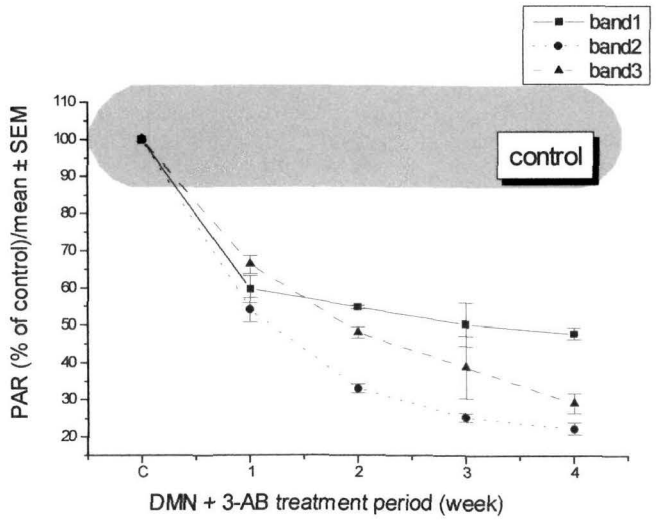


Figure 42. Relationship between PAR of cellular protein bands b1, b2 and b3 of spleen cells and DMN + 3-AB exposure period as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1 - protein of molecular weight 43 kDa, Band 2 (group of proteins) proteins of molecular weight 20-14.3 kDa (likely to be histones) and Band 3 - protein of molecular weight < 7 kDa (Fig. 34 C).

III) BMC:

Band 1: Following similar strategy, the PAR of BMC protein band b1 (Fig. 34 B) was quantified densitometrically (§ 2.26.) in control and DMN + 3-AB treated mice. The results are tabulated below (Table XXVIII).

Band 2: Densitometric quantification (§ 2.26.) was done similarly for band b2 (Fig. 34 B) (Table XXIX).

Table XXVIII: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					P ≤ 0.01	P ≤ 0.05
0	100	100	100	100 ± 0.00		
1	65.69	59.00	65.56	63.41 ± 2.20	S	S
2	45.20	38.47	38.96	40.87 ± 2.16	S	S
3	31.25	35.48	25.57	30.76 ± 2.87	S	S
4	29.32	21.73	20.32	23.79 ± 2.79	S	S

Table XXIX: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	85.45	81.08	79.10	81.87 ± 1.87	NS	S
2	15.16	18.64	20.58	18.12 ± 1.58	S	S
3	2.92	3.87	2.52	3.10 ± 0.40	S	S
4	1.23	2.03	3.07	2.11 ± 0.5	S	S

Band 3: Steps similar to other cases were followed to quantify (§ 2.26.) band 3 (b3, Fig. 34 B) as well. The result is shown in Table XXX below.

Table XXX: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	93.09	82.58	84.08	86.58 ± 3.28	NS	NS
2	20.87	25.01	33.79	26.55 ± 3.8	S	S
3	9.91	10.34	7.97	9.40 ± 0.72	S	S
4	8.82	13.62	9.90	10.78 ± 1.45	S	S

In Fig. 43 a negative correlation was apparent between the PAR of protein bands b1, b2 and b3 of BMC and period of DMN + 3-AB treatment when compared with the control. The reduction was up to 80 % in band 1, over 95 % in band 2 and about 90 % in case of band 3 in the 4th week of treatment.

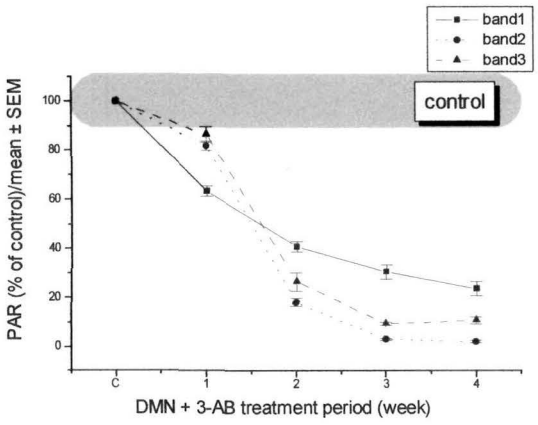


Figure 43. Relationship between PAR of cellular protein bands b1, b2 and b3 of BMC in control and DMN + 3-AB exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1- protein of molecular weight 43 kDa, Band 2 - protein of molecular weight 14.3 kDa (likely to be H2b) and Band 3 - protein of molecular weight < 7 kDa (Fig. 34 B).

IV) Blood lymphocytes:

Band 1: The PAR of protein band b1 (Fig. 34 D) of blood lymphocytes of control and DMN + 3-AB treated mice were similarly quantified densitometrically (§ 2.26.). The results of different experiments have been shown in Table XXXI.

Table XXXI: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					P≤0.01	P≤0.05
0	100	100	100	100 ± 0.00		
1	77.13	79.74	63.34	73.40 ± 5.08	NS	S
2	63.09	67.76	40.52	57.12 ± 8.41	NS	S
3	24.50	25.99	32.18	27.55 ± 2.35	S	S
4	17.74	18.07	20.99	18.93 ± 1.03	S	S

Band 2: Band 2, b2 (Fig. 34 D) was densitometrically quantified (§ 2.26.) in similar manner (Table XXXII).

Band 3: The steps of densitometric quantification (§ 2.26.) were followed for protein band b3 (Fig. 34 D) as in the earlier cases. The results are shown in Table XXXIII below.

Table XXXII: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					P≤0.01	P≤0.05
0	100	100	100	100 ± 0.00		
1	62.67	49.15	42.89	51.57 ± 5.80	N S	S
2	33.69	31.49	41.81	36.66 ± 3.31	S	S
3	28.17	26.34	32.00	28.83 ± 1.66	S	S
4	26.31	21.64	18.15	22.03 ± 2.36	S	S

Table XXXIII: Mean intensity or Pixel density of data of differences between control and DMN + 3-AB exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN + 3-AB treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					P≤0.01	P≤0.05
0	100	100	100	100 ± 0.00		
1	52.59	54.63	84.06	63.76 ± 10.10	NS	NS
2	48.16	48.45	67.84	54.81 ± 6.51	NS	S
3	47.57	31.15	56.55	45.09 ± 7.43	N S	S
4	29.06	24.09	42.66	31.93 ± 5.55	S	S

When the data were plotted in a graph, again a negative correlation was obtained between the PAR of protein bands b1, b2 and b3 of blood lymphocytes and period of

DMN + 3-AB treatment (Fig. 44). In band 1 the reduction was up to 80 % of control, in band 2, over 75 % and in band 3, about 70 % in the 4th week of treatment.

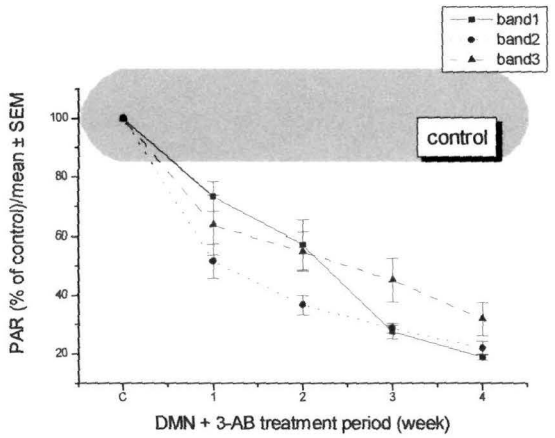


Figure 44. Relationship between PAR of cellular protein bands b1, b2 and b3 of blood lymphocytes of control and DMN + 3-AB treated mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1 - protein of molecular weight 20 kDa (likely to be H1), Band 2 - protein of molecular weight 14.3 kDa (likely to be H2b) and Band 3 - protein of molecular weight < 7 kDa (Fig. 34 D).

3.18.3. Histones after DMN exposure

I) Liver:

H1: Different immunodetection (§ 2.21.) results of PAR of histone H1 of liver (Fig. 35 A) of control and DMN exposed mice were quantified densitometrically (§ 2.26.). The data has been expressed as per cent (%) of control in term of mean ± SEM (Table XXXIV).

Table XXXIV: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Exp. 6 (%)	Mean ± SEM	Significance	
								$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100	100	100 ± 0.00		
1	73.23	68.88	85.55	61.45	69.45	75.45	72.83 ± 3.69	S	S
2	31.90	29.90	89.97	33.45	73.09	27.07	47.56 ± 10.99	S	S
3	35.81	40.80	64.44	31.07	53.44	25.55	41.85 ± 5.95	S	S
4	25.45	29.44	69.89	32.13	69.83	22.24	41.89 ± 9.07	S	S

H3/H2b: Steps similar to H1 were followed for the densitometric quantification (§ 2.26) of histone H3/H2b (Fig. 35 A). The results are shown in Table XXXV below.

Table XXXV: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Exp. 6 (%)	Mean ± SEM	Significance	
								$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100	100	100 ± 0.00		
1	89.10	92.86	86.46	93.67	81.89	94.87	89.80 ± 2.03	S	S
2	58.46	88.68	79.42	89.56	74.83	91.42	80.39 ± 5.11	NS	S
3	43.08	53.45	49.56	92.13	55.33	92.90	64.40 ± 9.05	NS	S
4	33.55	40.09	31.83	60.56	33.09	74.83	45.99 ± 7.19	S	S

H2a: Densitometric quantification (§ 2.26.) was done for histone H2a as well (Fig. 35 A) (Table XXXVI).

Table XXXVI: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Exp. 5 (%)	Exp. 6 (%)	Mean ± SEM	Significance	
								$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100	100	100 ± 0.00		
1	80.31	84.45	83.37	92.41	86.83	81.88	84.8 ± 1.75	S	S
2	66.01	51.60	53.43	88.86	89.81	69.41	69.85 ± 6.77	S	S
3	36.30	38.09	30.01	89.43	50.30	39.55	47.44 ± 8.81	S	S
4	22.24	20.72	25.44	50.43	52.03	26.29	32.85 ± 5.87	S	S

Fig. 45 below again reveals a negative correlation between the PAR of histone proteins of liver and DMN treatment period. There was up to 55 % reduction in the PAR level of H1, over 50 % reduction in H3/H2b and about little less than 70 % reduction in H2a in the 4th week of treatment in comparison with the control.

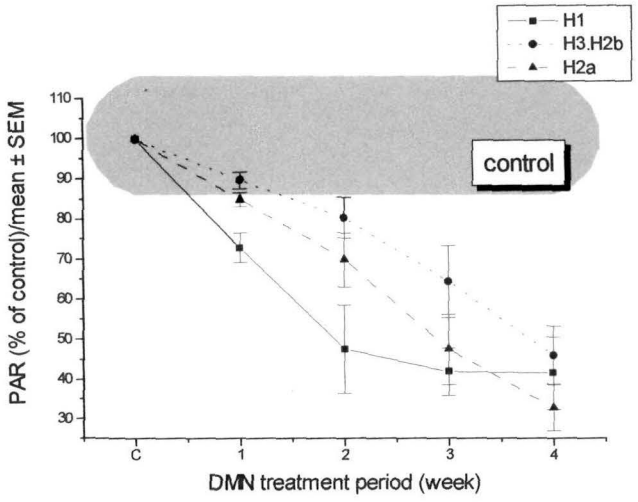


Figure 45. Relationship between PAR of liver histone proteins in control and DMN treated mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment.

II) Spleen:

H1: Results of immunodetection (§ 2.21.) for PAR of histone H1 of SC in control and DMN treated samples were quantified densitometrically (§ 2.26.) as done in case of liver histones (Table XXXVII).

Table XXXVII: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	91.95	93.34	85.75	86.78	89.34 ± 1.93	NS	S
2	93.99	85.69	86.94	93.78	90.01 ± 2.20	N S	S
3	53.80	53.00	49.31	77.19	58.32 ± 6.36	S	S
4	26.36	25.47	25.63	49.85	31.82 ± 6.01	S	S

H3/H2b: Similarly, densitometric quantification (§ 2.26.) was carried for histones H3/H2b (Fig. 35 B). Table XXXVIII shows the results.

Table XXXVIII: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	89.05	86.98	88.14	69.28	83.36 ± 4.71	NS	S
2	41.08	38.87	64.64	53.23	49.45 ± 5.96	S	S
3	27.92	30.45	31.65	26.27	29.07 ± 1.21	S	S
4	23.67	25.45	36.39	25.88	27.84 ± 2.88	S	S

H2a: The usual strategy of densitometric quantification (§ 2.26.) was used here too Fig. 35 B). The results are given in Table XXXIX.

Table XXXIX: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Exp. 4 (%)	Mean ± SEM	Significance	
						$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100	100 ± 0.00		
1	79.05	81.56	75.86	80.89	79.34 ± 1.27	S	S
2	55.05	59.97	63.07	57.89	58.99 ± 1.69	S	S
3	23.05	30.05	29.06	31.43	28.39 ± 1.84	S	S
4	19.09	25.09	23.56	26.08	23.44 ± 1.53	S	S

Upon plotting the data in a graph again the characteristic negative correlation was observed between the level of PAR of different histones of SC and period of DMN treatment (Fig. 46). The reduction was over 65 %, over 70 % and about 80 % of control for histones H1, H3/H2b and H2a, respectively, in the 4th week of treatment.

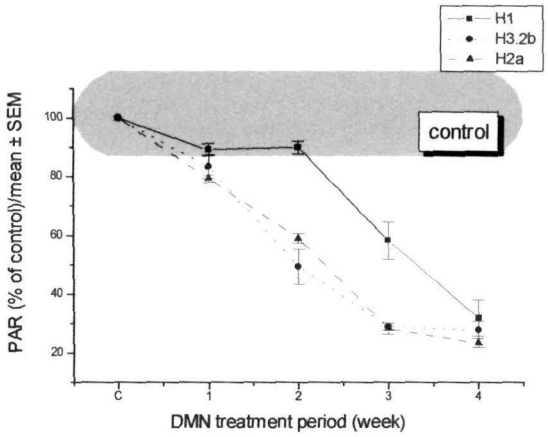


Figure 46. Relationship between PAR of histones of spleen cells in control and DMN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment.

III) Blood lymphocytes:

H1: Using similar strategy, the PAR of histone H1 of blood lymphocytes (Fig. 35 C) was quantified densitometrically (§ 2.26.) in control and DMN exposed mice. The results are summed up in Table XXXX.

Table XXXX: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	51.05	60.43	52.01	54.49 ± 2.97	S	S
2	50.77	58.35	51.75	53.62 ± 2.38	S	S
3	47.10	44.00	45.02	45.37 ± 0.91	S	S
4	46.16	43.96	45.86	45.32 ± 0.68	S	S

H3/H2b: Densitometric quantification (§ 2.26.) was done in similar way for histones H3/H2b (Fig. 35 C). The results are shown in Table XXXXI.

Table XXXXI: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	47.12	61.09	62.03	56.74 ± 4.82	NS	S
2	43.55	43.83	47.28	44.88 ± 1.19	S	S
3	36.49	50.66	55.85	47.66 ± 5.78	NS	S
4	27.06	49.88	50.04	42.32 ± 7.63	NS	S

H2a: Similarly, densitometric quantification (§ 2.26.) was done for histone H2a (Fig. 35 C). The results are tabulated below (Table XXXXII).

Table XXXXII: Mean intensity or Pixel density of data of differences between control and DMN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of DMN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	62.33	50.49	55.63	56.15 ± 3.42	S	S
2	31.15	28.15	30.91	30.07 ± 0.96	S	S
3	50.03	30.02	52.72	44.25 ± 7.16	NS	S
4	45.66	32.44	49.31	42.47 ± 5.12	S	S

Again, the negative correlation between the PAR of histone proteins of blood lymphocytes and DMN exposure period was apparent (Fig. 47). There was up to 50 % reduction in the PAR of H1, over 55 % reduction in H3/H2b and H2a in 4 week treatment groups when compared with the control.

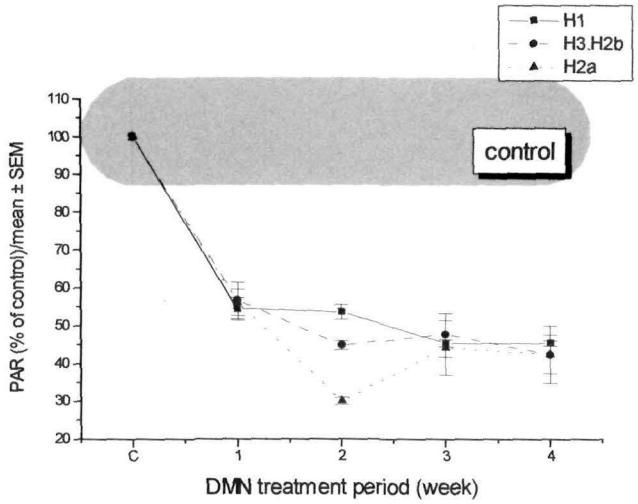


Figure 47. Relationship between PAR of blood lymphocyte histone proteins of control and DMN treated mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment.

3.18.4. Influence of AEBN

I) Spleen:

Band 1: Band intensities of cellular protein bands b1 (Fig. 36 A) of SC in control and AEBN treated mice was obtained. The data has been expressed as per cent (%) of control in term of mean ± SEM (Table XXXXIII).

Table XXXXIII: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	97.39	99.55	99.99	98.97 ± 0.80	NS	NS
2	20.77	19.48	21.53	20.59 ± 0.59	S	S
3	20.35	19.48	21.28	20.37 ± 0.51	S	S
4	20.35	18.86	21.33	20.18 ± 0.71	S	S

Band 2: Similarly, band 2, b2 (Fig. 36 A) was also quantified (§ 2.26.) (Table XXXXIV).

Table XXXXIV: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	96.02	95.83	97.82	96.56 ± 0.63	NS	S
2	30.63	37.13	30.35	32.70 ± 2.21	S	S
3	14.39	13.60	15.26	14.41 ± 0.47	S	S
4	14.23	12.83	15.23	14.09 ± 0.69	S	S

The plot of data in Fig. 48 shows a negative correlation between the PAR of protein bands b1 and b2 of SC and AEBN treatment period. There was up to 80 % reduction in the PAR level of band 1 and about 90 % reduction in band 2 in the 4th week of treatment in comparison with the control.

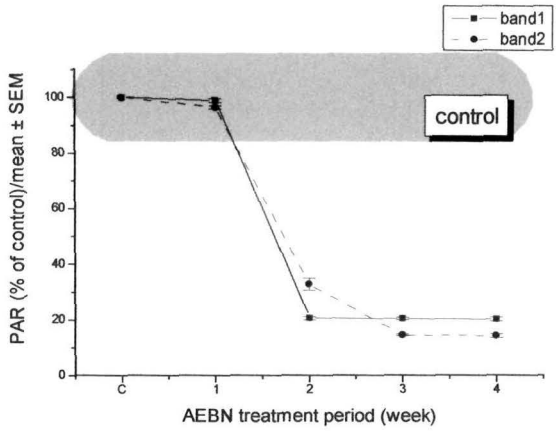


Figure 48. Relationship between PAR of cellular protein bands b1 and b2 of spleen cells in control and AEBN treated mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1 - protein of molecular weight 20 kDa (likely to be H1) and Band 2 - protein of molecular weight < 14.5 kDa (likely to be H2a) (Fig. 36 A).

II) BMC:

Band 1: Results of immunodetection experiments (§ 2.21.) for PAR of protein band b1 of BMC (Fig. 36 B) in control and AEBN treated samples (§ 2.16.) were quantified densitometrically (§ 2.26.) as done in case of spleen. The results are shown in Table XXXXV.

Table XXXXV: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	85.10	76.79	83.18	81.69 ± 2.51	NS	S
2	32.46	29.47	30.85	30.92 ± 0.86	S	S
3	44.23	58.91	40.86	48.00 ± 5.54	NS	S
4	43.32	56.45	40.01	46.59 ± 5.02	S	S

Band 2: Densitometric quantification (§ 2.26.) was also done for protein band b2 (Fig. 36 B) (Table XXXXVI).

Table XXXXVI: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	91.58	47.17	43.08	60.61 ± 15.5	NS	N S
2	20.07	62.23	61.16	47.82 ± 13.87	NS	NS
3	35.77	52.02	49.18	45.65 ± 5.01	S	S
4	16.89	25.18	15.14	19.07 ± 3.09	S	S

Upon plotting the data, the negative correlation between the PAR of protein bands b1 and b2 of BMC and period of AEBN treatment was observed even in this case (Fig. 49). The reduction in PAR level was up to 50 % of control in band 1 and over 80 % in case of band 2 in the 4^{th} week of treatment.

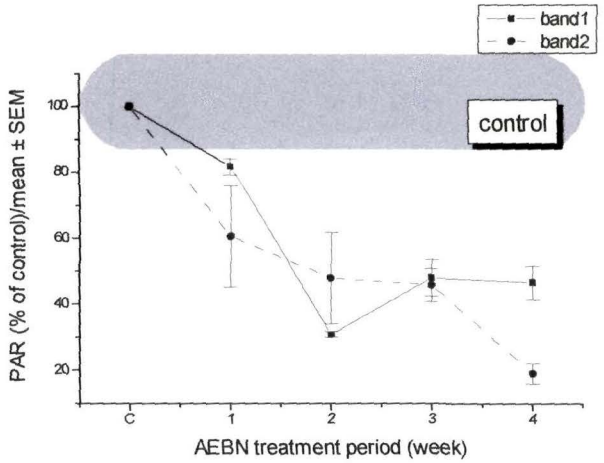


Figure 49. Relationship between PAR of cellular protein bands b1 and b2 of BMC in control and AEBN treated mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1 - protein of molecular weight 68 kDa and Band 2 - protein of molecular weight 14.3 kDa (likely to be H2b) (Fig. 36 B).

III) Blood lymphocytes:

Band1: Following similar strategy, the PAR of cellular protein band b1 (Fig. 36 C) of blood lymphocytes was quantified densitometrically (§ 2.26.). The results are summed up in Table XXXXVII.

Band 2: Band 2, b2 (Fig. 36 C) was quantified densitometrically as done in other cases. The results are shown in Table XXXXVIII below.

Table XXXXVII: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	83.11	73.19	80.56	79.95 ± 2.97	NS	S
2	73.63	72.60	74.72	73.65 ± 0.61	S	S
3	73.24	67.06	58.08	66.12 ± 4.40	NS	S
4	41.43	40.89	52.00	44.77 ± 3.61	S	S

Table XXXXVIII: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' means the difference is significant.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	78.19	74.19	77.55	76.64 ± 1.24	S	S
2	66.06	61.98	61.19	63.07 ± 1.50	S	S
3	63.33	61.57	50.35	58.41 ± 4.06	S	S
4	43.02	50.74	42.01	45.25 ± 2.75	S	S

Band 3: This band, b3 (Fig. 36 C) was also quantified (§ 2.26.) in similar way. The results are summed up in Table XXXXIX.

Table XXXXIX: Mean intensity or Pixel density of data of differences between control and AEBN exposed groups expressed as % of control. (0), 1, 2, 3 and 4 – period of AEBN treatment in week; t-test was carried out to find out the significance of difference between the mean of the data of each treatment group with respect to the mean of control at $P \leq 0.01$ and $P \leq 0.05$. 'S' or 'NS' means the difference is significant or not significant, respectively.

Period of treatment (week)	Exp. 1 (%)	Exp. 2 (%)	Exp. 3 (%)	Mean ± SEM	Significance	
					$P \leq 0.01$	$P \leq 0.05$
0	100	100	100	100 ± 0.00		
1	98.32	99.29	53.50	83.70 ± 15.10	NS	S
2	79.71	82.83	50.61	71.05 ± 10.25	NS	NS
3	94.37	79.04	37.39	70.26 ± 17.02	NS	NS
4	52.87	40.02	33.33	42.07 ± 5.73	S	S

The plot of data (Fig. 50) reveals the negative correlation between the PAR of protein bands b1, b2 and b3 of blood lymphocytes and period of AEBN exposure, as in other tissues. There was up to 55 % reduction in the PAR level of band 1 and band 2 and about 60 % reduction in band 3 in the 4th week of treatment in comparison with the control.

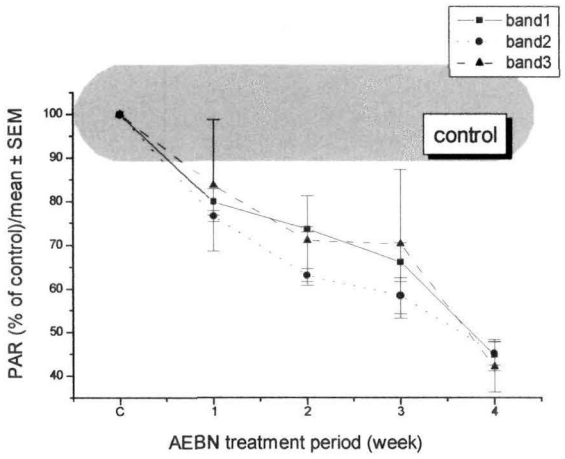


Figure 50. Relationship between PAR of cellular protein bands b1, b2 and b3 of blood lymphocytes in control and AEBN exposed mice as revealed by the mean intensity (pixel density) (% of control) after densitometric quantification of the immunodetected Western blots. Graph was plotted using PAR (% of control)/mean ± SEM vs. period of treatment. Band 1 - protein of molecular weight 68 kDa, Band 2 - protein of molecular weight 43 kDa and Band 3 - protein of molecular weight 14.3 kDa (likely to be H2b) (Fig. 36 C).

Chapter 4

DISCUSSION

There are several prospective of studying carcinogenesis since carcinogenic transformation is a highly complex and multi-step process. It is known that the process involves several events at molecular level including mutations of different kinds, gene rearrangements, gene amplifications, altered gene expressions, *etc.* to name the most obvious. These events occur during initiation, promotion and progression phases (Pitot, 1986). The repair systems, inherent in living beings, can potentially alter all the molecular events listed above. Thus, they exercise great influence on the transformation process in both human and animal models (Tomelette & Pfeifer, 1994), thereby, adding further complexity. In addition, an ever increasing number of chemicals, many types of radiations, numerous error in the normal cellular processes governing life, large number of identified oncogenes, *etc.* have also been implicated as causes of cancer. A process so diverse as cancer, therefore, remains uncontrolled despite advancement of knowledge base and technology. It is becoming more obvious now than earlier that the fight has to continue. We have not been able to zero down on the exact unified mechanism(s) by which cellular transformations are initiated and maintained essentially due to this extreme diversity of possibilities and causes. It has also not been possible to design a drug to mitigate suffering of individuals with different types pf cancers. If that had happened, cancer would have been one of the diseases of the past like cholera, plague, tuberculosis, *etc.* These diseases of the past caused rampant loss of human lives, today they stand almost completely eradicated from the face of earth.

Therefore, there is a growing agreement among scientists and medical professionals of recent time that while the search for exact mechanisms of cellular transformation should continue, one must focus on early detection of onset of cellular transformation in order to increase the clinical efficacy of the present medical protocols. It has been postulated that such an approach can effectively increase the cancer therapeutic index. The present medical technology and tests are able to detect cancer at a later stage when it is too late to do much to help the individual. It is, therefore, of utmost urgency to find suitable 'biomarkers', which can act as a general, convenient and easy tool for detection of different types of cancers (Cuzick, 1999; Levi, 1999). A large group of scientists across the globe are working on finding or developing a convenient and easy way to detect cancer at an early stage. By doing so even the present medical intervention protocols can reduce human cancer related mortality and morbidity by an estimated 35 % (Levi, 1999). Presently there are several tests that are employed to establish existence of cancer; none is conclusive (Ladik and Forner, 1994). In other words, the biomarkers of cancers currently in use are only indicative. Some of them are also expensive and cumbersome involving use of sophisticated instruments and technological know-how. The existing technology is helping to some extent the human population in developed countries, which also have better medical infrastructure and smaller populations. In countries like India and other developing or under-developed countries the scenario is very different. Most, especially rural population, do not have access to good medical infrastructure and the population density is high. Keeping these realities in mind, this piece of work was designed as a step in the direction of identifying a suitable biomarker of cancer that should be simple, sensitive and easy to apply as a tool for early detection of cancer and for mass screening.

Molecular biomarkers have such potentials as the control of all metabolic activities lies in genes located on the genome or chromosomes. Abnormal functions of the

genes or loss of control of their activities mediate cellular transformation as is evident from the studies relating to oncogenes or tumor suppressor genes (Kahn and Graf, 1986). Direct assessment of such events is likely to serve as the best biomarker for cancer detection. However, again use of molecular probes to directly detect such abnormalities of gene(s) is highly complex requiring sophisticated laboratory set ups and manpower besides being very expensive. These limitations make it difficult to apply such approaches for cancer detection in developing or under-developed countries. Furthermore, the metabolic abnormalities emancipating from such changes in gene(s) manifest their influences on metabolic processes through their products, proteins or enzymes. The abnormal gene product is the main cause of cellular transformation since they exercise the control of metabolic functions. Thus, it would be more pertinent if the search for such abnormal metabolic agent is made which can be used as biomarker for early detection of cancer. With this in mind, the work embodied in this thesis took up the probing of abnormality of gene(s) on chromosomes by biochemical characterization of such gene products and the metabolic pathway(s) controlled by the product(s). In this, poly ADP-ribosylation (PAR) of cellular proteins, including chromosomal proteins (histones), was chosen because of compelling evidences of its relevance to cellular transformation (Althaus and Richter, 1987; Boulikas, 1990; Boulikas *et al.*, 1990; Sharan *et al.*, 1998).

With this in background, the work of this thesis was designed to monitor gene(s) on chromosomes at biochemical level in order to characterize the chromosomes/genome during cellular transformation induced by chemical carcinogens. PAR of cellular proteins (total proteins as well as chromosomal proteins) has been taken as the main biological end-point of this study. The main gene(s) regulating this important metabolic process is located on chromosome 14 in mouse (MGI, 2003), the model used for the investigation as detailed in § 1.2. Cancer being an *in vivo* event, the study used a protocol of exposing normal healthy mice to carcinogens for up to 4 weeks to monitor the events of transformation essentially during initiation stage of transformation. The usefulness of mouse as experimental animal for research relating to cancer is high and the results of such investigations can be easily extrapolable to human situation (Berns, 1999). As has been described earlier, initiation is an irreversible but very important stage of transformation where cells commit to transformation (Pitot, 1986; Dorr and Koeller, 1994). Two qualitatively different chemical carcinogens were chosen for the work. The target specific hepatocarcinogen, dimethylnitrosamine (DMN) is a potent carcinogen causing liver transformation (Magee and Barnes, 1956; Montessano and Barsch, 1976; Pariat and Sharan, 1995; 1998). The dose of DMN used in this study has been shown to actually cause cellular transformation in mice liver (Pariat and Sharan, 1995). A general suspected natural carcinogen, aqueous extract of betel nut (AEBN), was also taken for the study due to its relevance to a large section of population across the world, especially in the north-eastern part of India (IARC, 1985; 1987; Sharan, 1996). It is to be noted that AEBN is not reported to have any specific target organ for transformation unlike DMN, which specifically causes liver cancer. Nonetheless, a very high population index of cancer among betel nut chewers makes it an important cancer-causing agent. Since, the study used PAR of proteins as the biological end-point, a known chemical inhibitor of this metabolic process, 3-aminobenzamide (3-AB) was also utilized in the study as positive control (Sims *et al.*, 1982; Banasik and Ueda, 1994).

4.1. STRUCTURAL CHARACTERIZATION

Structural architecture and integrity of chromosomes or genomic DNA is critical (Hancock and Boulikas, 1982). The integrity of the superstructure of chromosome has direct bearing on the functionality of gene(s) located on them (Poirier *et al.*, 1982; Aubin *et al.*, 1983; Boulikas, 1987; Bellard *et al.*, 1989; Nagao *et al.*, 1991; Saikia *et al.*, 1999). The structural integrity of the genomic DNA can be conveniently monitored by DNase I induced fragmentation of DNA (Margison and O'Conner, 1979; Schneeweiss *et al.*, 2000). DNase I randomly cleaves DNA, which is directly proportional to accessibility of the DNA to DNase I (Bellard *et al.*, 1989). Therefore, if the DNA is in a condensed state, access of DNase I to DNA is limited and *vice-versa*. Consequently, limited or extensive fragmentation of genomic DNA shall result in its condensed or relaxed states, respectively (Margison and O'Conner, 1979; Schneeweiss *et al.*, 2000). This can be conveniently visualized after the DNase I fragmented genomic DNA is electrophoresed on an agarose gel. This approach has been utilized in the initial experiments to follow the influence of DMN and AEBN on mice during initial 4 weeks of exposure. Fig. 5 shows that the DMN enhanced the susceptibility of genomic DNA isolated from spleen cells of mice toward DNase I digestion. An increasing degradation of genomic DNA was observed as DMN exposure increased. The genomic DNA isolated from SC of mice exposed to DMN for 4 weeks (lane 4, Fig. 5) was virtually totally degraded by DNase I under the conditions of experiment used in this study (§ 2.7.). The genomic DNA is heterogenous as evident in lane CU (Fig. 5). However, certain stretches of DNA in the genome of untreated mice are accessible to DNase I in normal case also. This is shown as the large genomic DNA got fragmented to pieces of DNA of appox. size range of 10 to 60 kDa (lane C, Fig. 5). However, upon exposure to DMN, the genomic DNA seemed to undergo relaxation starting from 1st week itself and the process continued till the end of our observation period (lanes 2 to 4, Fig. 5). Thus, there was a general and consistent pattern of increase in the degree of degradation of DNA. These observations indicate that genomic DNA underwent relaxation almost immediately following exposure to DMN *in vivo*. DNA has highly complex and super-coiled structure in the normal physiological condition (Hancock and Boulikas, 1982). In this conformation DNA is normally inaccessible or slightly accessible to various chemicals/agents and nucleases for interaction. This is very important in order to maintain the integrity of such vital macromolecules that govern the very existence of the cell or the living system. The normal status of the DNA is unchanged as long as any external agents like chemicals, radiations, *etc.* or internal agents such as the free radicals do not force it to do so.

This relaxation of genomic DNA caused by DMN, as revealed by the ease with which DNase I progressively fragmented it, seems to be enhanced when 3-AB was concurrently administered on mice chronically exposed to DMN *in vivo*. In Fig. 6, it is evident that there was further increase in the degree of DNase I digestion of genomic DNA isolated from spleen cells of mice exposed to DMN + 3-AB. The relaxation of the genome was maximal in the 3rd and 4th weeks of treatment. Comparing Fig. 5 (DMN alone) with Fig. 6 (DMN + 3-AB) it stands out that the genomic DNA of mice undergoes faster relaxation when DMN is administered concurrently with 3-AB. Virtually entire genome was fragmented to pieces significantly smaller than 8.2 kDa in the 4th week. It can, therefore, be concluded that DMN, in presence of 3-AB, caused faster relaxation of the DNA conformation.

The introduction of pulse-field gel electrophoresis (PFGE) has revolutionized the way to analyze large DNA pieces, hallmark of eukaryotes, to get additional insights. PFGE can resolve DNA pieces on agarose gel in the size range of 30-50 kbp to well over 10 Mbp (Schwartz and Cantor, 1984). This was also used in the investigation to further our understanding on the genomic DNA isolated from DMN and DMN + 3-AB groups of mice. Following DNase I fragmentation, the samples were loaded for PFGE. The CU lane (Fig. 8) shows two large sized bands of DNA. Exposure to DMN caused progressive degradation of both these bands, which was relatively stronger for the lower band (lanes CU, C, 1 & 2, Fig. 8). In the 4^{th} week, the entire genome was reduced to very small pieces of DNA by DNase I, which looked like a smear (Fig. 8). The result is in conformation with the agarose gel electrophoresis result (Fig. 5) and suggests that the smaller sized fragment of genomic DNA was the initial target of DMN interaction.

A general carcinogen, aqueous extract of betel but (AEBN), was also used in this study to follow its effects on the structural characteristics of genomic DNA or total chromosomes. AEBN, like DMN, also affected the structural organization of genomic DNA of spleen cells of mice chronically exposed to AEBN (Fig. 7). However, its effect was moderate as compared to DMN. AEBN also caused relaxation and opening up of DNA as evident by progressive ease of fragmentation by DNase I. However, the intensity was relatively less. The accessibility of DNase I to DNA was noticeable from 3^{rd} week onwards of AEBN exposure (lanes 3 & 4, Fig. 7).

In our experiments evidence has been recorded of DMN, either alone or in conjunction with 3-AB, or AEBN induced change in the superstructure of genomic DNA or chromatin (Figs. 5-8). The super-structure underwent relaxation in both the case with different intensities. Structural change in genome has serious consequences on the functionality of DNA (Hancock and Boulikas, 1982; Schneeweiss *et al.*, 2000). A progressively relaxed state of genomic DNA is taken as indicative of consequent higher transcribing or active genes (Margison and O'Conner, 1979). It has been shown earlier that carcinogenesis is favored when genes shift to relatively higher state of transcription (active genes) (Saikia *et al.*, 1999; Schneeweiss *et al.*, 2000). The results presented in this thesis also show that DMN pushed genes to progressively active state (Figs. 5, 8) and in presence of 3-AB the effect of DMN was further potentiated (Fig. 6). Even upon exposure to AEBN the effect was similar albeit less (Fig. 7). The dose of DMN used in this investigation is reported to cause hepatocarcinogenesis (Pariat and Sharan, 1995; 1998; 2002) and in that situation the genomic DNA of liver was in a relaxed state (Pariat and Sharan, 1998; 2002). The results (Figs. 5-8) show that even spleen cell genomic DNA was moved into relaxed state under the influence of DMN. It is possible that several genes of spleen cells also, including proto-oncogenes(s), were activated. This may means that initiation of transformation might have been made even in spleen cells after DMN or AEBN exposure. Some reports in literature indicate that qualitatively different carcinogens may have some molecular commonality during initiation stage (Magee and Barnes, 1956; Pariat and Sharan, 1998; 2002). This seems logical since during initiation the activated carcinogen interacts with gene(s) to cause mutations of different kind (Gabridge and Legator, 1969; Guttenplan *et al.*, 1976; Pegg *et al.*, 1978). Both DMN and AEBN have been reported to be metabolically activated or changed from potential carcinogen to ultimate carcinogen in mice when administered *in vivo*

(Magee and Barnes, 1956; Guttenplan *et al.*, 1976; Lijinsky, 1976; Ashby *et al.*, 1979; Rao and Das, 1989; Sharan, 1996; Saikia *et al.*, 1999). An earlier study has shown that AEBN, at comparative dose induced DNA strand breaks in mouse kidney cells *in vitro* and enhanced rate of cell division (Wary and Sharan, 1988). A relatively higher dose was found to be toxic to kidney cell *in vitro* (Wary and Sharan, 1988). Moreover it has also been shown by Ames test that mutation frequency was significantly enhanced by AEBN (Balachandran and Sharan, 1998). AEBN was also found to cause unscheduled DNA synthesis suggesting damages to genomic DNA (Sharan and Wary, 1992). Once they interact with gene(s) the normal cellular processes are likely to be altered.

4.2. BIOCHEMICAL CHARACTERIZATION

Further molecular characterization of the genome (e.g. structural alterations, gene activation, gene amplification, *etc.*) involves use of specific gene probes. The outcome of such investigation, though very useful, shall have serious limitations on its applied potential (e. g., for mass screening of population for early detection of cancer) due to more than one reason. To start with molecular probes shall be needed which are expensive and fragile limiting the affordability to a smaller segment of the population especially in poorer countries. In addition, skilled manpower and sophisticated laboratory set-up shall be necessary to perform the assay for population screening. Thus, its application will be difficult in ordinary medical clinics and primary health centers in countries like India. Therefore, biochemical approach was chosen which could overcome practically all difficulties listed above. It is expected that the applied potential of the biochemical approach shall be significantly high even in developing and under-developed countries.

For the biochemical characterization, the biological end-point of status of poly ADP-ribosylation (PAR) of cellular proteins was chosen for many obvious reasons. Correlations between the cellular PAR and different cellular and molecular events including carcinogenesis have been observed in our laboratory and elsewhere (Benjamin and Gill, 1980; Althaus *et al.*, 1995; Bhatia *et al.*, 1990; Alderson, 1990; Moss and Vaughan, 1990; Boulikas, 1991; 1993; Saikia, 1996; Saikia *et al.*, 1998; 1999; Pariat and Sharan, 1998; 2002; Devi, 2001). This post-translational modification of mainly chromosomal proteins, unlike other known post-translational modification, causes drastic changes in protein-DNA interaction essential to structural integrity of chromosomes (Hancock and Boulikas, 1982; Althaus, 1992; Aubin *et al.*, 1983; Boulikas, 1991; Schneeweiss *et al.*, 1995; Sharan *et al.*, 1996; 1998a). Since indication of significant structural changes has been recorded in mouse genome following *in vivo* exposure of mice to DMN and AEBN (Figs. 5-8), it is prudent that we choose PAR as the biological end-point. Essentially, monitoring PAR of cellular proteins indirectly measures the activities of the two main genes controlling cellular PAR reaction. One of the two genes, poly ADP-ribose polymerase (PARP) gene, has been mapped on chromosome 14 of mice (Fig. 2) while the other, poly ADP-ribosyl glycohydrolase (PARG) gene, is proposed to be on the same chromosome. Their gene products catalyze PAR and the level of PAR is outcome of activities of these gene products (Althaus *et al.*, 1995; Sharan *et al.*, 1996). PAR was monitored by ELISA based immuno-probe assay, developed in our laboratory (Sharan *et al.*, 1998b). This assay was chosen because of its sensitivity, reproducibility and the ease with which it could be used (Sharan *et al.*, 1998b). Unlike the commonly used radioactive method

(Schneeweiss *et al.*, 1995), the immuno-probe assay was totally safe from possible ill effects of radiation. The assay involved use of rabbit polyclonal antibodies (Ab) raised in the laboratory, the 1st Ab, against heterogeneous ADP-ribose polymer isolated from normal (control) mouse spleen cells (§ 2.11.). Commercially available anti-rabbit IgG tagged with alkaline phosphatase (ALP) was used as the 2nd Ab. Since the 1st Ab was raised against heterogeneous ADP-ribose polymers and not against any particular mouse proteins, the Ab worked as a general probe for all poly ADP-ribosylated cellular proteins. This gives a tremendous advantage to the assay employed in this work since any cellular protein carrying ADP-ribose polymer as a post-translational modification on it could be detected. The ALP conjugated to the 2nd Ab helped developed a distinct purple coloration of protein bands on slot- or Western blot. This could be easily quantified either by densitometry or by digital photography using appropriate software.

The obvious first task was to determine the immunogenicity of the 1st Ab and its specificity to detect ADP-ribose polymers on any protein. The immunogenicity was tested by Ouchterlony immunodiffusion assay (§ 2.12.). Fig. 9 shows a characteristic precipitin line appearing only between the wells containing the isolated spleen histone proteins (the Ag) and the 1st Ab. No precipitin line developed between BSA and Ab confirming the immunogenicity of the 1st Ab used for the investigation and also its specificity. The specificity of the 1st Ab against poly ADP-ribose was confirmed using SVP (§ 2.13.). As SVP very exclusively degrades ADP-ribose polymer from any protein (Sugimura, 1974), in principle, no detection of PAR should be made when a protein is totally free of ADP-ribose polymer. As is evident in Fig. 10 the color intensity of slots of histone proteins isolated from mouse blood lymphocytes was progressively diminished with increasing time of SVP digestion of the histone proteins before slotting (lanes 0 - 3, Fig. 10). There was no detectable color band after 30 min of SVP digestion of histones (lanes 4-6, Fig. 10). Equal amounts o histone proteins were loaded on each slot. Thus, these results (Figs. 9, 10) confirm that the 1st Ab used in the investigation was highly immunogenic and very specific to ADP-ribose polymers.

Using the immuno-probe assay, status of total PAR of all cellular proteins as well as individual, mainly histone, proteins were monitored for up to 4 weeks in diverse tissues (liver, spleen cells (SC), bone marrow cells (BMC) and blood lymphocytes) of mice chronically exposed to a hepatocarcinogen, DMN, or a general, naturally occurring carcinogen, AEBN *in vivo*. DMN is a nitroso derivative of aliphatic hydrocarbons and has been shown to induce hepatocarcinogenesis (Magee and Barnes, 1956; Pariat and Sharan, 1995). Similarly, strong association of AEBN with human carcinogenesis has been suspected (IARC, 1985; 1987; Sharan, 1996). It has also been shown earlier that initiation of carcinogenesis in mice generally occurs within 4 weeks or so (Pariat and Sharan, 1998; 2002). Therefore, the period of investigation in this piece of work was limited to 4 weeks. The choice of liver, SC, BMC and blood lymphocytes for monitoring PAR of proteins were made to test applicability of the assay in diverse tissues of mice with different physiological states. While liver is a solid, multicellular and pre-mitotically fixed tissue, SC, BMC and blood lymphocytes are not so. Further, SC and BMC are heterogeneous cell population of differentiating cells while blood lymphocytes are homogeneous differentiated cell population. Blood lymphocytes were chosen for the work for an additional important reason. From the viewpoint of possible applied use of results in

screening of population for early detection of cancer, blood lymphocyte is a very convenient tissue. Drawing of blood from a subject requires minimal medical intervention and causes virtually no trauma. If blood lymphocytes mirror biochemical and physiological status of a subject, use of biopsies could be totally avoided in the future. As we know, present medical practices heavily depend on analysis of biopsies especially in cancer diagnosis. There is not only surgical intervention and trauma involved in the process of obtaining biopsies; there are ethical questions too associated with it. Therefore, investigation using blood lymphocytes assumes further significance.

DMN progressively lowered the total PAR of cellular proteins in different tissues investigated. This lowering was seen on total PAR of cellular proteins (lanes II, Fig. 11) by slot-blot immuno-probe assay (§ 2.18. & 2.21.). This was observed while the total protein slotted remained essentially constant as evident by the ink stained (§ 2.22.) replica slots (lanes I, Fig. 11). Even the protein profile of these tissues did not show any noticeable visible changes (Fig. 29) suggesting that during the period of observation quantitative changes in cellular proteins did not occur. Table I - IV and Figs. 15 - 18 show the quantification results of total PAR of proteins in liver, SC, BMC and blood lymphocyte, respectively. The quantification data on total PAR (Tables I - IV) and plots (Figs. 15-18) confirm the visual impression emancipating from the immunoprobed slots (Fig. 11). In general, there was a near-linear but inverse relationship between the periods of DMN exposure and the total PAR of cellular proteins in all the tissues examined. The lowering was also statistically significant in most case, especially after 2 weeks of DMN exposure to mice *in vivo* (Tables I - IV). While liver, SC and BMC showed the maximum inhibition of total PAR, over 60 % in 4^{th} week as compared to the control (Tables I - III), blood lymphocytes exhibited about 45 % inhibition during the same period (Table IV). The effect of AEBN, the other general carcinogen used in this investigation, was examined in SC, BMC and blood lymphocyte only (Fig. 14). Total PAR of cellular proteins also showed similar trends after quantification with tissue specific variations (Figs. 26 - 28) while the proteins slotted for immunoprobe assay (lanes I, Fig. 14) or the proteins profile (Fig. 32) were essentially invariant. Except in SC, the lowering of total PAR was statistically significant as compared to the respective controls (Tables XII - XIV). The effect of AEBN exposure on total PAR of BMC proteins was maximal in 4^{th} week (reduced to nearly 75 % of the control) (Table XIII). The effects of AEBN exposure in SC (Table XII) and blood lymphocytes (Table XIV) were comparable to that in case of DMN (Tables II & IV). The results, therefore, suggest that both carcinogens, irrespective of their chemical differences or tissue specificities, lowered total PAR of cellular proteins, thereby inducing qualitative differences as exposure to the carcinogens progressed up to 4 weeks *in vivo*.

3-Aminobenzamide (3-AB) is a potent chemical inhibitor of PARP (Purnell and Whish, 1980a; Christine and Bernard, 1987; Banasik and Ueda, 1994). Therefore, it shall also exercise influence on the cellular PAR reactions (Rankin *et al.*, 1989; Purnell and Whish, 1980a; Devi, 2001). To dwell further into the biochemical process, 3-AB was acutely administered to mice simultaneously with chronic, low-dose DMN exposure. Slots of samples from DMN + 3-AB exposed mice (Fig. 12) show expected further reduction in total PAR of cellular proteins. Quantification of the total PAR of proteins of liver (Table V; Fig. 19), SC (Table VI; Fig. 20), BMC (Table VII; Fig. 21) and blood lymphocytes (Table VIII; Fig. 22) make the point

obvious. With the exception of liver, SC, BMC and blood lymphocytes exhibited higher extent of inhibition of total PAR of cellular proteins following concurrent exposure of mice to DMN + 3-AB than only DMN. It is, however, not clear why liver, which is the target organ for DMN induced carcinogenesis, did not reproduce the trend (compare Tables I & V), rather showed in opposite. This point shall be investigated in the future. Nonetheless, the extent of lowering of total PAR of SC, BMC and blood lymphocyte proteins was further accentuated by the concurrent presence of 3-AB. In case of blood lymphocytes, which showed minimal lowering effect of DMN on total PAR of proteins compared to liver, SC and BMC, the combined exposure of DMN + 3-AB caused significantly more effect on PAR (compare Tables IV & VIII). The level of PAR at the 4th week of DMN administration (Table IV) went down from 54.41 ±6.53 to 28.39 ±2.32 (Table VIII). This is expected since the concentration of 3-AB used in this investigation (2 mM) is reported to inactivate PARP *in vivo* (Christine and Bernard, 1987; Devi, 2001). As has been recorded, DMN also caused lowering of total PAR of proteins. Thus, together, they are likely to cause higher order of lowering of total PAR of cellular proteins. This suggests that DMN, a known hepatocarcinogen, and 3-AB, a chemical inhibitor of PARP, work in the same direction as far as PAR reaction is concerned. From this viewpoint, chemical inhibition of PAR reaction is likely to create a similar biochemical situation in a living system to that by DMN. Together they will simply aggravate the situation further.

Since the main target proteins for PAR is reported to be chromosomal proteins (Althaus *et al.*, 1995; Miwa and Sugimura, 1990; Sharan *et al.*, 1998a; Pariat and Sharan, 1998; Devi, 2001; Kun *et al.*, 2002), especially histone proteins (Boulikas, 1990; Schneeweiss *et al.*, 1995; Sharan *et al.*, 1996; 1998a; Pariat and Sharan, 1998), the effects of DMN and AEBN were also monitored on the total PAR of histone proteins isolated form liver, SC and blood lymphocytes of mice exposed to the carcinogens *in vivo*. The isolated histone proteins (§ 2.17.) were slot-blotted on NC membrane and immuno-probed. Since only histone proteins were slotted, the resulting PAR signal shall indicate the state of poly ADP-ribosylation of histone proteins exclusively. The result of slot-blot immuno-assay to measure total PAR of all histone proteins isolated from liver, SC and blood lymphocyte of mice exposed to DMN is shown in Fig. 13, lanes II while lane I shows the replica slot stained for total histone proteins by India ink. Their quantification data have been shown in Tables IX, X & XI, respectively. It is evident that the general trend of lowering of total PAR of cellular proteins with progression of DMN exposure period was essentially mirrored by the plot of total PAR of isolated histones vs. DMN exposure period (Figs. 23 – 25). The plot for liver (Fig. 23), however, showed some resistance to the lowering effect of DMN on the total PAR of histones in the 2nd and 3rd weeks of DMN exposure making the effect statistically non-significant (Table IX). Barring this, all reductions of total PAR of histone proteins in liver, SC and blood lymphocytes were statistically significant at $p \leq 0.05$ and 0.01 as compared to their respective controls (Tables IX – XI). While this qualitative change was recorded on total histone proteins, there was no evidence of quantitative changes in histone proteins under the influence of DMN exposure. The profile of histone proteins isolated from liver, SC and blood lymphocytes exhibited no apparent differences between the controls and treated samples (Fig. 31). Thus, it can be concluded that DMN induced qualitative changes in histone proteins by way of reducing their extents of poly ADP-ribosylations during the initiation stage of DMN induced carcinogenesis.

To analyze the PAR of individual histones, the isolated histone protein mixture from liver, SC and BMC of mice exposed to DMN was electrophoresed by SDS-PAGE, Western blotted the resolved histone proteins on NC membrane and immuno-probed (panel I, Fig. 35). A replica blot was stained by India ink to visualize all proteins resolved. Different histone proteins have been identified (panel II, Fig. 35). It is evident from Fig. 35 that most histones of liver, SC or blood lymphocytes were poly ADP-ribosylated and showed similar and expected trend of lowering of PAR with period of DMN exposure. Quantitative plot of PAR for liver (Fig. 45), SC (Fig. 46) and blood lymphocytes (Fig. 47) reaffirm the observation. The isolated histone proteins have been identified and, for the purpose of quantitative analysis, grouped into three categories, namely H1, H3/H2b and H2a in this study. This was done based on their migration on PAGE gel and convenience of quantification (Fig. 35). While the PAR of histone H1 reduced to about 45 % of control in the 4th week of DMN treatment in liver (Tables XXXIV) and blood lymphocytes (Table XXXX), SC showed relatively more effect reaching nearly 32 % of the control (Table XXXVI). Histones H3/H2b also follows the same general pattern of diminution of PAR showing less effect in liver (Tables XXXV) and blood lymphocytes and more effect in SC (Table XXXVIII). Histone H2a also repeated the pattern exhibiting differential PAR in the three tissues examined (Tables XXXVI, XXXIX and XXXXII for liver, SC and blood lymphocyte, respectively). The plots of these data are given in Figs. 45, 46 and 47 for liver, SC and blood lymphocytes, respectively. The general conclusion that may be arrived at is that PAR differentially modifies different histone proteins in different tissues. This is in line with earlier reports of the same nature (Schneeweiss *et al.*, 1995; Sharan *et al.*, 1996). The histone proteins of SC seemed to be relatively more modified by PAR under the influence of DMN while the target organ for DMN induced transformation, liver, as well as blood lymphocytes showed lower extent of PAR of its histone proteins under the influence of DMN. There will be need of further investigation to clarify the reason for this differential effect of DMN.

By comparing the levels of total PAR of cellular proteins and that of total histone proteins, it is evident that the major target of cellular PAR are histone proteins. This is in line with earlier reports that almost 90 % of total cellular PAR is contributed by the PAR of different histone proteins (Boulikas, 1990; Boulikas *et al.*, 1990; Schneeweiss et al., 1995; Sharan *et al.*, 1998a; Pariat and Sharan, 1998; Devi, 2001), making histones the most preferred target proteins for PAR. Qualitatively different extents of PAR of histones shall influence its weak-interactions with DNA to organize the chromatin superstructure (Boulikas, 1991). Since it was shown that the chromatin underwent relaxation under similar conditions (Figs. 5, 8), it appears that lowering of total PAR of histone proteins may have facilitated the relaxation of the chromatin organization.

Considering the predominance of histone proteins in PAR reaction and consequent effect of this reaction on the structural/functional organization of chromatin, it was desirable to observe the PAR of individual cellular proteins in the whole homogenate (WH) of tissues obtained from mice exposed either to DMN or AEBN. To achieve this, cellular proteins in the WH of tissues were first resolved on 12 % SDS-PAGE gels (§ 2.19.). The resolved proteins on the gels were transferred onto NC membrane by Western blotting (§ 2.20.). The Western blot was immuno-probed with the 1st Ab followed by 2nd Ab and the bands containing poly ADP-ribosylated proteins were

color developed (§ 2.21.). As in case of slot blots, the protein bands on the Western blots were also quantified. A replica Western blot was simultaneously stained with India ink to ascertain the amounts of proteins loaded in each lane (§ 2.22.). The immuno-assay, being specific to poly ADP-ribose modification and not to any particular cellular protein, should detect all poly ADP-ribosylated cellular protein bands on a Western blot. In this, if histones are the preferred targets for PAR, one may expect that mainly histone proteins would be detected on a Western blot subjected to the immuno-assay. This was indeed observed.

The Western blots of WH of liver, SC, BMC and blood lymphocytes of mice exposed to DMN *in vivo* are shown in Fig. 33. Results of identical experiment for AEBN exposure are shown in Fig. 36. In both the cases, the left panel (I) has been immuno-probed while the right panel (II), a replica Western blot, was stained with India ink. As expected, there was no noticeable quantitative difference in the amount of proteins blotted for all four tissues examined as revealed by ink stained blots (panels II, Figs. 33 and 36). However, upon immuno-probing, the qualitative difference between individual proteins in terms of poly ADP-ribosylation was apparent as DMN (panels I, Fig. 33) or AEBN (panels I, Fig. 36) exposure periods progressed. It was again apparent that histone proteins were the major target proteins for PAR among the WH proteins of different tissues. Under the influence of AEBN, PAR of individual proteins immuno-probed on Western blots showed a slightly different picture in SC, BMC and blood lymphocytes (Fig. 36). While histone proteins still appeared to be the main target proteins for PAR, unlike DMN, the bands were not so distinct.

Few high molecular weight proteins also seemed to be poly ADP-ribosylated (Figs. 33 and 36). It is known that PARP, molecular weight range 90 – 120 kDa (Kameshita *et al.*, 1984; Shizuta *et al.*, 1985), is among other non-histone protein, which is also a favored target of PAR (Althaus and Richter, 1987; Adamietz, 1987). This has also been shown earlier in studies using radioactive assay using ^{32}P-NAD^{+} and cell lines *in vitro* (Schneeweiss *et al.*, 1995; Sharan *et al.*, 1996; 1998a). This process of self-modification of PARP is called autoribosylation or automodification. Automodification is proposed to be a regulatory mechanism for regulation of PARP enzyme activity (Althaus and Richter, 1987; Adamietz, 1987; Althaus, 1992; Althaus *et al.*, 1995). Increasing automodification renders PARP progressively inactive and *vice versa*. In AEBN exposed group, high molecular weight poly ADP-ribosylated proteins appeared more like diffused bands in all three tissues (panels I, Fig. 36). In addition, some other relatively low molecular weight cellular proteins were also modified. In this, an important and relevant class of non-histone proteins is high mobility group (HMG) proteins. The HMG proteins have also been reported to be modified by PAR under the influence of DMN (Pariat and Sharan, 1998; 2002). These are the protein bands besides histones and PARP that are visible on the Western blots after immuno-probing (panels I, Figs. 33 and 36). Western blots of isolated histone proteins from liver, SC and blood lymphocytes of mice exposed to DMN upon immuno-probing showed almost similar patterns and visual impression of lowering of PAR of histones with extension of DMN exposure period was obvious (Fig. 35). It has to be noted that isolated histone proteins resolve into different histones quite differently by SDS-PAGE when compared to resolution of cellular proteins from WH. Being highly basic and free from other cellular proteins, the profile of isolated histones looks different.

The concurrent presence of 3-AB with DMN, on expected lines, further significantly inhibited cellular PAR reaction as evident from the Western blots of WH of liver, SC, BMC and blood lymphocytes of mice exposed *in vivo* to DMN + 3-AB (Fig. 34). Lanes loaded with samples from 2nd week onwards of exposure showed rather faint PAR signals (panels I, Fig. 34) while the right replica panels II, showed that almost equal amount of proteins were loaded for Western blotting. This is in confirmation with the results shown earlier for the total PAR of cellular proteins wherein also the same was observed when 3-AB was simultaneously administered on mice along with DMN (Fig. 12).

Visual impression of immuno-probed Western blots of WH showed the expected pattern of progressive lowering of PAR of individual proteins with progression of DMN or AEBN exposure periods. For further detailed quantitative analysis, two to three protein bands of the immuno-probed Western blots showing PAR modifications of proteins in different tissues were quantified. Two PAR modified protein bands were chosen from the immuno-probed Western blot of liver WH of mice exposed to DMN for analysis (Fig. 33, B). Protein band 1 (b1; mol. wt. 68 kDa) and 2 (b2; mol. wt. < 7 kDa) upon PAR quantitative analysis showed generally significant decline in PAR modification with progression of DMN exposure period (Tables XV - XVI, Fig. 37). In the WH proteins of SC from DMN exposed mice (Fig. 33, A), protein bands 1 and 2 (b1 and b2, Fig. 38) showed clear progressive reduction in the level of PAR (Tables XVII and XVIII). These proteins have not been identified but protein band 1 (mol. wt. 20 kDa) and 2 (mol. wt. 14.3 kDa) are likely to be histones H1 (mol. wt. 21.1 kDa) and H2b (mol. wt. 14.5 kDa), respectively, due to molecular weight similarities. Both these proteins showed significant reduction in their PAR modification with increasing DMN exposure period. AEBN exposure affected PAR of these two protein bands even more (Tables, XXXXIII and XXXXIV, Fig. 48). In BMC from DMN exposed mice, PAR of protein band 1 (b1; mol. wt. 14.3 kDa), likely to be histone H2b (mol. wt. 14.5 kDa) and a small molecular weight protein (b2; mol. wt. < 7 kDa) showed significant reduction in their PAR (Tables XIX and XX, Fig. 39). Blood lymphocytes from DMN exposed mice showed similar results for protein bands 1 (b1; mol. wt. 43 kDa) and 2 (b2; mol. wt. 14.3 kDa), likely to be histone H2b (mol. wt. 14.5 kDa) (Tables XXI and XXII, Fig. 40). The WH proteins from SC (Tables XXXXIII - XXXXIV), BMC (Tables XXXXV - XXXXVI) and blood lymphocytes (Tables XXXXVII - XXXXIX) of mice exposed to AEBN also repeated the observations for selected protein bands (Figs. 48 – 50, respectively). Simultaneous administration of 3-AB with DMN again produced results on expected lines for selected protein bands that were quantified from liver (Tables XXIII – XXIV), SC (Tables XXV – XXVII), BMC (Tables XXVIII – XXX) and blood lymphocytes (Tables XXXI – XXXIII) as evident in Figs. 41 – 44, respectively. In all, different tissues of mice exposed to either DMN or AEBN for up to 4 weeks exhibited expected declining but differential PAR of different cellular proteins. These WH proteins undergoing PAR modification in different tissues are likely to include histones H1, H3/H2b, H2a, H4 and some other proteins as apparent from analysis done above.

Reduction in the extent of poly ADP-ribosylation of particularly histone proteins has serious biological consequences. The first impact of this would be change in the structural organization of DNA since the charge status of histones shall be altered. Histones form an integral part in normal organization of DNA to form chromatin. As

is evident from DNase I degradation of genomic DNA or chromatin, the superstructure underwent progressive relaxation (Figs. 5-8) coinciding with decrease in the extent of PAR modification of different, mainly histone, proteins. The alteration naturally shall have impact on the functional status of gene on chromosomes (Poirier *et al.*, 1982; Aubin *et al.*, 1983; Boulikas, 1987; Bellard *et al.*, 1989; Nagao *et al.*, 1991; Saikia *et al.*, 1999; FaraoneMennella *et al.*, 2000). The level of PAR that has been assayed and quantified in this investigation is the result of the interplay of two main gene products involved in PAR metabolism, PARP and PARG (Sharan *et al.*, 1996). These genes are proposed to be located on chromosome 14 in mouse (Figs. 1 and 2; MGI, 2003). The total genome of mouse underwent progressive relaxation (Figs. 5-8). Thus, it can be proposed that at least selected segments of chromosome 14 had undergone alterations in a way that the expressions of PARP and PARG genes were altered. In general a lowering of PAR of cellular proteins was observed in all cases. This may be caused by (a) low expression of PARP gene or (b) less active or inhibited PARP enzyme activity. Alternately, when (a) expression of PARG genes is more or (b) the PARG enzyme is more active then also the same will happen. This investigation did not address to these issues because one major purpose of this investigation was to look into the applied potential of PAR immuno-assay. However, the total outcome of any or all of these possibilities translated into lowering of extent of PAR of cellular proteins. This provides a valuable support to application of the results of the investigation for welfare of human kind.

4.3. Significance of the lymphocyte proteins results in light of DMN and DMN + 3-AB treatment

Early detection of cancer continues to be a formidable challenge to the scientific community and search for a suitable biomarker for use in mass screening of population is of utmost importance in our fight against cancer (Doll and Feto, 1981; Pelomaki *et al.*, 1993; Doll and Koeller, 1994; Cuzick, 1999; Levi, 1999; Srivastava, 1999). Constant efforts are being made worldwide to find an effective and reliable biomarker and a convenient assay for early detection of cancers, especially in its initiation stage, which is when a cell gets committed to transformation. A good biomarker must be one, which could be common to all cancers. This broadens the scope of use of this marker for detection of any cancer. Poly ADP-ribosylation (PAR) of proteins meets the criterion. A decade of research in our laboratory had set a firm foundation of PAR of cellular proteins as an eminently suitable biomarker for detection of cancer (Sharan *et al.*, 1998b). As elaborated earlier in this thesis, this post-translational modification of cellular proteins alters the charge and conformational status of the modified protein. The reaction is reversible. The target proteins include several chromosomal proteins/enzymes, including histone proteins, relating to chromatin organization and its function. The structural organization and functional state of chromatin, therefore, is postulated to be strongly influenced by PAR. The hallmarks of carcinogenesis include molecular events associated with the functionality of genes. Altered gene expression, accelerated or diminished gene activities and mutations are some such events associated with carcinogenesis. Since, PAR of chromosomal proteins also alters the functionality of chromosomes, the association of PAR of chromosomal proteins and carcinogenesis has a sound basis. In the strategy adopted in this piece of work for early detection of cancer, the level of PAR modification of cellular proteins has been assessed. An almost linear but negative correlation has been found between PAR of cellular proteins and initiation of

carcinogenesis by DMN and AEBN in a mouse model. It is to be noted that the immuno-probing assay was employed in the investigation to detect PAR of proteins irrespective of on which proteins the modification has been affected.

Usually for detection of cancer a confirmatory test comes when the biopsies of cancerous tissues are investigated. Obtaining biopsies involves several medical and ethical issues and poses problems when a patient is subjected to surgical procedures to obtain biopsies. Further, obtaining biopsies of some tissues (e. g., brain tumor) is a serious medical intervention besides being traumatic to a patient. In this piece of work embodied in the thesis this issue has been addressed to directly. Besides tissues like liver, spleen cells (SC) and bone marrow cells (BMC), blood lymphocytes were used for the investigation after the mice was exposed to DMN or AEBN in a chronic exposure protocol *in vivo*. The aim was to find out if any correlation existed between the level of PAR of blood lymphocyte proteins and initiation of cancer. Simultaneously, the same was tested in liver, SC and BMC which also served as positive controls for each other. Obtaining blood from any patient and separating blood lymphocytes are routine medical practices involving virtually no medical intervention, post-procedure medical care or trauma to a patient. Both drawing of blood from a patient and separating blood lymphocytes are again very routine matters requiring very basic and minimal medical infrastructures. The working hypothesis was that if a correlation was found between PAR of blood lymphocyte proteins and carcinogenesis then, in future, medical screening might only involve drawing of blood from a patient for screening. This also makes it possible to handle a large number of patients at ordinary hospitals and primary health centers and even para-medics shall be able to do this.

All tissues examined in this investigation have shown a negative, nearly straight line, correlation between period of DMN or AEBN exposures and PAR of proteins from different tissues. The most significant observation made in this study that has direct implication on its use in medical practice is that even blood lymphocytes exhibited significant reduction in PAR modification of cellular proteins with progression of DMN or AEBN exposure periods. This was in line with the trend exhibited by liver, SC and BMC. Both total PAR of cellular proteins and PAR of individual histone proteins showed similar trends. Different tissues or proteins did show variability in terms of PAR, but the general message was clear. Tissue specific variations were found which is normally expected. Though obtaining liver, SC or BMC from mice is no problem but the same is not true for human situation. The results from mouse blood lymphocytes give convincing clues that the strategy can be easily extrapolated to human situation. Further, immuno-probe assay of total PAR of cellular proteins is relatively simple as compared to the assay of PAR of individual proteins. The former involves slotting the sample directly on a NC membrane (slot-blot) and immuno-probing while, the latter, requires electrophoresis, transblotting the proteins from gel onto a NC membrane (Western blotting) and then immuno-probing. Since our results show a very clear, reproducible and statistically significant correlation between total PAR of blood lymphocyte proteins and initiation of carcinogenesis induced by two different classes of chemical carcinogens, DMN and AEBN, (Figs. 11, 13 and 14), it is logical to postulate that this slot-blot immuno-probe assay can be employed in human situation also. It is postulated, based on the results presented in the thesis using mouse model that this strategy shall apply to any type of human cancer as well.

The biochemicals required for the whole slot-blot immuno-probe assay can, in principle, be packaged into a kit making it very convenient for transport. Most of the constituents of this kit have good shelf-life and require ordinary refrigeration, *etc.* The 1st Ab is the critical constituent which actually detects the PAR modifications of proteins. In the investigation, the 1st Ab was raised against heterogeneous ADP-ribose polymer obtained from mouse SC. Preliminary investigation on human blood lymphocytes using the same 1st Ab against mouse ADP-ribose polymer shows that the 1st Ab was also able to detect PAR of proteins in human blood lymphocytes. This already supports the basic premise of application of the assay for detection of human cancer and for its use as a tool of mass screening. However, all procedures shall need to be standardized for assay involving human samples before final conclusion is drawn. Much remains to be done before it is applied to detect human cancer. Nonetheless, the work carried out and elaborated in the thesis takes us closer to the goal of making cancer detection simple, sensitive and reliable in the near future.

Chapter 5

CONCLUSION

Using a hepatocarcinogen, dimethylnitrosamine (DMN), and a naturally occurring general carcinogen, aqueous extract of betel nut (AEBN), in a chronic *in vivo* administration protocol on Swiss albino mice, this work has attempted to characterize changes on the genome or chromosomes of the mice during initiation stage of carcinogenesis. DMN has been shown to initiate cancer in mice in about 4 weeks of exposure. The characterization of the chromosomes was done by monitoring structural changes in the mouse genome using DNase I. Further characterization was done at biochemical level by monitoring poly ADP-ribosylation (PAR) of cellular proteins by an ELISA-based immuno-probe assay developed in the laboratory. Total PAR of cellular proteins as well as PAR of individual proteins, including isolated histone proteins, were monitored in liver, spleen cells (SC), bone marrow cells (BMC) and blood lymphocytes. The extent of total PAR of cellular proteins as well as that of histones was quantified densitometrically.

☼ The mouse SC genome underwent progressive relaxation starting from 2nd week of DMN or AEBN administration as indicated by results of DNase I digestion of the genome. Analysis done by agarose gel electrophoresis and pulse-field gel electrophoresis produced similar, confirmatory results.

☼ DMN caused relatively more relaxation of the genomic DNA than AEBN under similar conditions.

☼ Concurrent administration of 3-aminobenzamide (3-AB), an inhibitor of PAR reaction, with DMN further relaxed the genome suggesting involvement of PAR in the process.

☼ For biochemical characterization, PAR of cellular proteins was monitored using a novel ELISA-based immuno-probe assay developed in the laboratory. The assay is simple, sensitive, reliable, cost-effective and environment friendly since it does not involve use of any radioisotope. Total PAR of cellular proteins was assayed by slot-blot immuno-probing while PAR of individual proteins was assayed by Western-blot immuno-probing.

☼ The hepatocarcinogen DMN affected the total PAR of cellular proteins in liver, the target organ, as well as other tissues such as SC, BMC and blood lymphocytes. AEBN also showed similar results in all cases.

☼ Statistically significant and progressive lowering of total PAR of cellular proteins in the four tissues studied was caused by DMN or AEBN almost immediately after exposure of mice to the carcinogen in a chromic oral administration protocol.

☼ DMN in combination with 3-AB, on expected lines, further inhibited the total PAR of cellular proteins in all tissues examined.

☼ Tissue specific differences were apparent and the extents of inhibition of total PAR of cellular proteins were different in different tissues and for the two carcinogens used in this investigation.

☼ Histones proteins were found to be the main target proteins for PAR in all tissues beside some other high and low molecular weight proteins.

☼ Histone proteins were grouped as H1, H3/H2b and H2a for the analysis. PAR of these histones were variable in different tissues. However, in all cases, DMN or AEBN lowered the extent of their PAR progressively during initiation stage of carcinogenesis.

☼ Concurrent presence of 3-AB with DMN further lowered the PAR of histone proteins on expected lines. This suggests that DMN or AEBN affected gene activities or enzyme activities of PAR reaction in a way that PAR was lowered.

☼ Blood lymphocytes mirrored the effect for both DMN and AEBN in terms of total PAR of cellular proteins as well as PAR of individual proteins, particularly histones. Lowering of PAR was statistically significant in all cases beyond 2nd week of administration.

☼ The effect of two different carcinogens, DMN and AEBN, on the PAR of blood lymphocyte proteins may provided us with a handy tool for monitoring biochemical or physiological status of individuals exposed to carcinogens. This may possibly be a good biomarker for early detection of cancer or for monitoring progress of carcinogenesis since (1) it is a common post-translational modification associated with many proteins and (2) the assay, employed in this investigation, detects only the ADP-ribose moieties and not a particular protein.

☼ It is envisaged that immuno-probe assay of PAR can be applied for mass screening of cancer. Blood lymphocytes can be easily prepared for the immuno-probe assay.

☼ The slot-blot immuno-assay can potentially be used in ordinary laboratory set up by even semi-skilled persons.

Chapter 6

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